On the origin of nitrous oxide and its oxygen
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Dorien M. Kool
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With references, with summaries in English and Dutch

Abstract

Nitrous oxide (N₂O) is a greenhouse gas that contributes to global warming and the destruction of stratospheric ozone. To reduce N₂O emissions to the atmosphere it is important to understand how and where it is produced. This research aimed to identify the presence and importance of an hitherto elusive N₂O production pathway named ‘nitrifier denitrification’. The potential of this pathway had been identified in pure cultures, but experimental proof of the presence of nitrifier denitrification in actual soils remained inconclusive due to the lack of adequate methodology.

A promising approach to distinguish nitrifier denitrification from other N₂O production pathways (nitrification and denitrification) was based on tracing the stable isotopes of oxygen (O) and nitrogen (N) in N₂O. However, this approach did not account for the effect of O exchange between H₂O and intermediates of the N₂O production pathways. Our literature review suggests that such O exchange may likely be present in soil and aquatic environments. In soil incubation experiments using O and N tracing, we showed that O exchange can indeed strongly determine the O isotopic composition of N₂O. We quantified O exchange for denitrification of NO₃⁻ to N₂O, and deduced that O can occur during nitrifier pathways of N₂O production as well. Next to N₂O, we demonstrated that the O isotopic signature of NO₃⁻ in soil could also be affected by O exchange.

Accounting for O exchange, we subsequently developed a novel dual isotope approach to study N₂O production pathways in soil. We therewith showed for the first time that nitrifier denitrification can indeed be a production pathway of N₂O in soils. We further studied how environmental controls of N₂O may affect the individual pathways differently, and showed that nitrifier denitrification responds idiosyncratically to soil moisture content.

In conclusion, the revealed significance of O exchange between H₂O and intermediates of N₂O production in soil has serious implications for source determination of N₂O and NO₃⁻ in ecosystems. The acknowledgement of nitrifier denitrification as distinct N₂O production pathway in soil is an important step forward in our understanding of N₂O production to ultimately obtain accurate inventories and effective mitigation strategies for N₂O emissions.
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Chapter 1

General Introduction
Nitrous oxide in our environment

Despite its popular name (laughing gas), nitrous oxide (N\textsubscript{2}O) is a serious matter. The contribution of atmospheric N\textsubscript{2}O to global warming and its ability to breakdown stratospheric ozone (Crutzen, 1981; Duxbury et al., 1993) are of great concern to our environment. Alarmingly, atmospheric concentrations of N\textsubscript{2}O have been and still are steadily rising since the start of the industrial era. Nitrous oxide has now become the prime ozone depleting emission (Ravishankara et al., 2009) and the third most important anthropogenic greenhouse gas (IPCC, 2007), with a global warming potential approximately 300 times higher than CO\textsubscript{2} (Ramaswamy et al., 2001). Evidently, the continuous increase in N\textsubscript{2}O emissions to our atmosphere constitutes a major environmental concern. To stabilize the current atmospheric concentrations of N\textsubscript{2}O, emissions would need to be reduced by about 50% (IPCC, 2007). Adequate mitigation of these emissions is only possible if we understand the processes that produce N\textsubscript{2}O. In other words, studying the origin of N\textsubscript{2}O is of global environmental concern.

![Global sources of N\textsubscript{2}O to the atmosphere](image)

Figure 1.1: Global anthropogenic and natural sources of nitrous oxide (N\textsubscript{2}O) to the atmosphere (total budget: 17.7 Tg N yr\textsuperscript{-1}). Soils (in grey compartments) globally comprise the largest source of N\textsubscript{2}O (IPCC, 2007; Ravishankara et al., 2009).
Nitrogen cycling and N\textsubscript{2}O production in soils

Nitrous oxide is produced through several processes in the nitrogen (N) cycle, related to the cycling of reactive N. Reactive N refers to organic and inorganic forms of N that are biologically, photochemically and/or radiatively active, in contrast to the vast but inert atmospheric dinitrogen (N\textsubscript{2}) pool (Galloway et al., 2008). Globally, soils comprise the largest of all anthropogenic and natural sources of N\textsubscript{2}O to the atmosphere (Figure 1.1) (IPCC, 2007; Ravishankara et al., 2009). Reactive N enters the soil through atmospheric deposition, fertilizer and manure applications, plant residues, and biological nitrogen (N\textsubscript{2}) fixation (Figure 1.2). Organic N can be broken down by microorganisms to (inorganic) ammonium (NH\textsubscript{4}+) through ‘mineralization’. This is an important step in making organic N available for plants and microorganisms. Microorganisms can take up NH\textsubscript{4}+ and convert it to nitrite (NO\textsubscript{2}) and nitrate (NO\textsubscript{3}) by ‘nitrification’. Through ‘denitrification’, microorganism turn NO\textsubscript{3} again to (gaseous) N\textsubscript{2} (Figure 1.2).

Nitrous oxide can be formed through several biochemical processes. In soils, nitrification and denitrification are conventionally considered as the prime N\textsubscript{2}O production processes (Figure 1.2) (Mosier et al., 1998b; Pérez et al., 2001). Nitrification is carried out by autotrophic bacteria that (i) oxidize ammonia (NH\textsubscript{3}, in equilibrium with NH\textsubscript{4}+) via hydroxylamine (NH\textsubscript{2}OH) to nitrite (NO\textsubscript{2}) (ammonia oxidizers), and (ii) oxidize NO\textsubscript{2} further to nitrate (NO\textsubscript{3}) (nitrite oxidizers) (Paul et al., 1996). During the first step of nitrification, N\textsubscript{2}O can be released as a by-product of ammonia oxidation (Hooper et al., 1979).
Denitrification is performed by heterotrophic bacteria that use NO$_3^-$ as electron acceptor when O$_2$ is not available. They reduce NO$_3^-$ via NO$_2^-$, nitric oxide (NO), and N$_2$O to N$_2$ (Knowles, 1982; Zumft, 1997). Nitrous oxide can be the end-product, and/or escape to the atmosphere as an intermediate compound before it is completely reduced to N$_2$.

To enable the development of accurate N$_2$O emission inventories and effective mitigation strategies for N$_2$O emissions, we need to understand how both total N$_2$O emissions and its individual production processes are affected by environmental factors, soil properties and land use. Main factors controlling total N$_2$O production in soil include aerobicity and related moisture content, carbon and nitrogen availability and pH (Knowles, 1982; Firestone et al., 1989; Paul et al., 1996; Robertson et al., 2007). However, the different pathways of N$_2$O production respond differently to these environmental factors. For example, where oxygen (O$_2$) is needed for nitrification, denitrification is inhibited by its presence (Knowles, 1982; Paul et al., 1996). Denitrification, as a heterotrophic process, requires an organic carbon source, and is therefore strongly dependent on the soil organic carbon (SOC) quality and C:N ratio (Knowles, 1982; Paul et al., 1996; Robertson et al., 2007). Autotrophic nitrifiers do not need organic C for their own metabolism. However, they are indirectly affected by C:N ratio and SOC quality through mineralization and immobilization rates that affect NH$_4^+$ availability, the most important factor regulating nitrification in soil (Paul et al., 1996; Robertson et al., 2007). Both nitrification and denitrification are favored by a relatively high pH with an optimum in the range of 7 to 8, but appear to respond to low pH differently (Knowles, 1982; Paul et al., 1996).

**Unconventional pathways of N$_2$O production: Nitrifier Denitrification**

However, the ‘conventional’ paradigm that considers (autotrophic) nitrification and (heterotrophic) denitrification as the two principal production pathways of N$_2$O is a simplified presentation of reality. It has long been acknowledged that a wide range of biological processes has the potential to produce N$_2$O as (by-) product, and that similar pathways may be carried out by various organisms. For example, nitrification may be carried out by heterotrophic organisms and methanotrophic bacteria as well; NO$_3^-$ may be subject to co-denitrification and
dissimilatory NO$_3^-$ reduction to ammonia; and several fungi and Archaea also capable of denitrification. In soils however, the relative contribution of most above mentioned N$_2$O production pathways is thought to be minor. Yet there is one other potential pathway that receives increasing interest in soil-based studies: the potential of autotrophic ammonia oxidizing bacteria (AOB) to produce N$_2$O through reduction of NO$_2^-$ (Figure 1.3). The terminology in literature has previously been inconsistent, but this process is now commonly labeled ‘nitrifier denitrification’ (Wrage et al., 2001).

Nitrifier denitrification was identified already four decades ago in pure culture studies (Hooper, 1968; Ritchie et al., 1972). However, despite this early discovery and continued pure culture studies (Poth et al., 1985; Remde et al., 1990; Zart et al., 1998; Colliver et al., 2000; Schmidt et al., 2004; Shaw et al., 2006), the proof that nitrifier denitrification can be a significant N$_2$O producing pathway in soil has remained elusive. Evidently, unraveling the potential of N$_2$O production through nitrifier denitrification in soil is vital to proper understanding of total N$_2$O production, as this distinct pathway will likely respond idiosyncratically to soil environmental conditions. Although literature acknowledges that ‘conventional’ nitrification would implicitly cover nitrifier denitrification as well (Granli et al., 1994; Mosier et al., 1998a; Hayatsu et al., 2008), nitrifier denitrification has not been experimentally distinguished from ‘nitrification’ in soil. Soil-based experimental studies increasingly suggest that nitrifier denitrification could contribute significantly to N$_2$O production in soil (Granli et al., 1994; Webster et al., 1996; Hütsch et al., 1999; Wrage et al., 2004b;
McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martín et al., 2008). However, conclusive proof of its actual occurrence in soil remains pending due to the lack of reliable analytical techniques.

Established methods to distinguish between sources of N$_2$O from soil have typically made use of nitrogen (N) isotope tracing (Stevens et al., 1997; Baggs et al., 2003; Tilsner et al., 2003; Bateman et al., 2005), and of specific inhibitors (Yoshinari et al., 1977; Robertson et al., 1987; Klemedtsson et al., 1988; Webster et al., 1996). Nitrogen isotope labeling techniques differentiate N$_2$O production from nitrification and denitrification in soil by applying and tracing $^{15}$N enrichment from NH$_4^+$ and NO$_3^-$. However, $^{15}$N labeling alone cannot distinguish the N$_2$O that results from NO$_2^-$ reduction (i.e. nitrifier denitrification) from the N$_2$O generated as by-product from ammonia oxidation (i.e. ‘conventional’ nitrification), as in both processes the N originates from NH$_4^+$ (Wrage et al., 2005; Hayatsu et al., 2008). Acetylene (C$_2$H$_2$) and O$_2$ have been used as inhibitors for specific (steps in) N$_2$O production processes, but unfortunately these inhibition techniques are not reliable as the targeted inhibition is not always complete and/or selective (Tilsner et al., 2003; Beaumont et al., 2004a; Beaumont et al., 2004b; Wrage et al., 2004b; Wrage et al., 2004a).

**The origin of N$_2$O and its oxygen**

To quantify the contribution of nitrifier denitrification to N$_2$O production, Wrage et al. (2005) proposed a novel ‘dual isotope approach’, based on tracing both the N and oxygen (O) isotopes in N$_2$O. The principle of this approach is that the origin of the O atom in the N$_2$O molecule would differ between production pathways (Figure 1.4). In the first step of ammonia oxidation (to NH$_2$OH), O is obtained from molecular oxygen (O$_2$). Nitrous oxide released as by-product of nitrification is therefore assumed to contain O derived from O$_2$. In the following oxidation steps of nitrification to NO$_2^-$ and NO$_3^-$, the added oxygen comes from water (H$_2$O). Correspondingly, N$_2$O from nitrifier denitrification would obtain 50% of its O from O$_2$ and the other 50% from H$_2$O, reflecting NO$_2^-$. The O in N$_2$O from ‘conventional’ denitrification would reflect that of NO$_3^-$, which in the case of nitrification-derived NO$_3^-$ would originate for 1/3rd from O$_2$ and 2/3rd from H$_2$O (Figure 1.4). Based on this principle, studying the origin of the O in N$_2$O could
thus improve our understanding of the processes responsible for its production.

In the novel dual isotope approach, soils are treated with $^{15}$N enriched NH$_4^+$ and NO$_3^-$ and $^{18}$O enriched H$_2$O. Evaluation of the isotopic enrichment of the produced N$_2$O will then identify the relative contribution of the different pathways to total N$_2$O production.

The origin and organization of this thesis

The general aim of the research presented in this thesis is to improve our understanding of the pathways of N$_2$O production in soil, and the pathway of nitrifier denitrification in particular. The dual isotope approach as proposed by Wrage et al. (2005) would be a fundamental tool for this work, developed to identify the contribution of nitrifier denitrification in soil for the first time. At the start, the main objectives were:

(i) To further develop the dual isotope labeling approach; which would enable
(ii) To quantify the relative contribution of nitrifier denitrification as pathway of N$_2$O production in soil; and
(iii) To study the idiosyncratic response to environmental controls of N$_2$O production through nitrifier denitrification.

However, shortly after the start of my PhD research, I found that the origin of the O in N$_2$O is more complex than assumed in the dual isotope approach of
The assumption that reaction stoichiometry determines the proportion of O in N₂O that is derived from O₂ and H₂O (Kendall, 1998; Pérez, 2005; Wrage et al., 2005) underestimated the significance of another process: O exchange between H₂O and intermediate compounds of the N₂O production pathways. Throughout this thesis, ‘oxygen (or O) exchange’ is used as short for the exchange of O between nitrogen oxides and H₂O. This finding necessitated closer investigation of the origin of the O in N₂O, which became a priority of my revised research objectives:

(i) To study, identify and quantify the process of O exchange between H₂O and intermediate compounds of the N₂O production pathways, and its effect on the O isotopic signature of N₂O produced in soil;
(ii) To develop and apply an advanced stable isotope tracing approach that accounts for the effect of O exchange and subsequently can identify nitrifier denitrification in soil-based studies; and
(iii) To study the significance and idiosyncratic character of nitrifier denitrification as production pathway of N₂O in soil.

This thesis presents my research on O and N isotope tracing to identify the origin of N₂O from soil. In chapter 2, I start with a literature review on O exchange between H₂O and intermediates of N₂O production processes. Subsequently, I present a series of experimental studies to unravel the process of O exchange and its effect on the origin of the O in N₂O. First, I identify the general presence of O exchange in soil, and quantify O exchange during denitrification (chapter 3). In chapter 4, I evaluate the occurrence of O exchange during the different pathways of N₂O production. Using the acquired knowledge on the process of O exchange, I then develop and apply an advanced dual isotope tracing approach. With this approach I show that, in soil, N₂O can indeed be produced through nitrifier denitrification (chapter 5). In chapter 6, I identify that nitrifier denitrification can contribute significantly to total N₂O production and that its relative contribution is idiosyncratically affected by moisture conditions. With an exploratory study presented in chapter 7, I demonstrate that O exchange may affect the isotopic signature of NO₃⁻ as well. In the final chapter 8, I summarize my main findings and discuss their implications for current and future research on the origin of N₂O.
Chapter 2

Oxygen exchange between (de)nitrification intermediates and H$_2$O and its implications for source determination of NO$_3^-$ and N$_2$O: a review

Abstract  Stable isotope analysis of oxygen (O) is increasingly used to determine the origin of nitrate (NO$_3^-$) and nitrous oxide (N$_2$O) in the environment. The assumption underlying these studies is that the $^{18}$O signature of NO$_3^-$ and N$_2$O provides information on the different O sources (O$_2$ and H$_2$O) during production of these compounds by various biochemical pathways. However, exchange of O atoms between H$_2$O and intermediates of the (de)nitrification pathways may change the isotopic signal and thereby bias its interpretation for source determination. Chemical exchange of O between H$_2$O and various nitrogenous oxides has been reported in the literature, but the probability and extent of its occurrence in terrestrial ecosystems remain unclear. Biochemical O exchange between H$_2$O and nitrogenous oxides, NO$_2^-$ in particular, has been reported for monocultures of many nitrifiers and denitrifiers that are abundant in nature, with exchange rates of up to 100%. Therefore, biochemical O exchange is likely to be important in most soil ecosystems, and should be taken into account in source determination studies. Failing to do so might lead to (i) an overestimation of nitrification as NO$_3^-$ source, and (ii) an overestimation of nitrifier denitrification and nitrification-coupled denitrification as N$_2$O production pathways. A method to quantify the rate and controls of biochemical O exchange in ecosystems is needed, and we argue this can only be done reliably with artificially enriched $^{18}$O compounds. We conclude that in N source determination studies, the O isotopic signature of especially N$_2$O should only be used with extreme caution.

Chapter 2

Introduction

Increased anthropogenic emissions of nitrate (NO$_3^-$) contribute to contamination of ground water and eutrophication of surface waters (Howarth et al., 1996; Galloway et al., 2003). Rising concentrations of nitrous oxide (N$_2$O) are of environmental concern because of its contribution to the greenhouse effect (Crutzen, 1981). Environmental legislation aimed at mitigating these emissions has resulted in increased attention on the study of their origin.

Stable isotope analyses of nitrogen (N) and oxygen (O) are increasingly used to quantify N transformations and to determine the original N sources of anthropogenic and naturally derived NO$_3^-$ and N$_2$O in the environment. Both the natural abundance variation of $^{15}$N and $^{18}$O and artificially enriched compounds are used to trace the sources of NO$_3^-$ or N$_2$O (Wahlen et al., 1985; Durka et al., 1994; Webster et al., 1996; Kendall, 1998; Mayer et al., 2002; Pérez et al., 2006).

Natural abundance studies utilize the fact that the various natural sources of NO$_3^-$ have distinct isotopic signatures (Figure 2.1). A wide range of $\delta^{18}$O values for atmospheric NO$_3^-$ have been reported (+15 to $+75\%_{SMOW}$, as summarized by Kendall). The $\delta^{18}$O of microbially produced NO$_3^-$ is partially determined by that of O$_2$, which contributes one O atom during ammonia oxidation to hydroxylamine (NH$_2$OH), and partially by that of H$_2$O, which contributes the other two O atoms during the further oxidation to nitrite (NO$_2^-$) and NO$_3^-$ (Figure 2)(Aleem et al., 1965; Hollócher et al., 1981; Andersson et al., 1983; Hollócher, 1984; Voerkelius, 1990; Kendall, 1998). Assuming that the $\delta^{18}$O of soil O$_2$ is approximately equivalent to atmospheric O$_2$ ($+23.5\%_{SMOW}$), and with $\delta^{18}$O of soil H$_2$O usually in the range of -25 to $+4\%_{SMOW}$ (Amberger et al., 1987), the $\delta^{18}$O of NO$_3^-$ formed by nitrification will range from -10 to $+10\%_{SMOW}$ (Pardo et al., 2004). However, the $\delta^{18}$O of O$_2$ in soil may be increased relative to atmospheric O$_2$ due to fractionation by respiration in soil (Lane et al., 1956; Guy et al., 1993; Kendall, 1998), resulting in higher $\delta^{18}$O values for NO$_3^-$ formed by nitrification (Kendall, 1998). Isotope fractionation during denitrification results in relative enrichment in $\delta^{15}$N and $\delta^{18}$O of the remaining NO$_3^-$ (Amberger et al., 1987; Böttcher et al., 1990; Aravena et al., 1998; Mengis et al., 1999; Mengis et al., 2001; Sebilo et al., 2006).

Microbial processes that produce and consume N$_2$O all tend to fractionate in favor of the lighter isotopes, leaving the residual compounds relatively enriched.
Oxygen exchange between (de)nitrification intermediates and H$_2$O: a review

In $\delta^{15}$N and $\delta^{18}$O (Handley et al., 1992; Bedard-Haughn et al., 2003). Various sources and pools of N$_2$O (soil, oceanic, tropospheric and stratospheric) therefore show distinct O isotopic signatures. Based on this natural abundance variation, the $\delta^{18}$O-N$_2$O signature is increasingly used in addition to $\delta^{15}$N-N$_2$O to characterize the source, production and consumption of N$_2$O (Wahlen et al., 1985; Kim et al., 1990; Yoshinari et al., 1997; Schmidt et al., 2004a; Van Groenigen et al., 2005a; Pérez et al., 2006).

Besides natural abundance methods, combinations of NH$_4^+$ and NO$_3^-$ that are artificially enriched with $^{15}$N are routinely used to study the processes of nitrification and denitrification and their relative contribution to N$_2$O production (Stevens et al., 1997; Panek et al., 2000; Baggs et al., 2003; Bateman et al., 2005). The additional use of $^{18}$O-enriched H$_2$O has been suggested to enable the distinction between N$_2$O from nitrification and nitrifier denitrification (the reduction of NO$_2^-$ to N$_2$O by nitrifiers) (Wrage et al., 2005). Similar to natural abundance studies, in this approach by Wrage et al. (2005) the different contributions of O$_2$-O and H$_2$O-O to N$_2$O-O in the various pathways is used for

Figure 2.1: Schematic overview of the N and O isotopic signature in NO$_3^-$ from different sources, after Kendall (1998). The arrow indicates the change in isotopic composition upon denitrification due to isotopic fractionation, of which the slope, $\delta^{15}$N:$\delta^{18}$O, is approximately 1:2 (Böttcher et al., 1990). Along the Y-axis the $\delta^{18}$O of atmospheric O$_2$ and (soil) H$_2$O are indicated.

in $\delta^{15}$N and $\delta^{18}$O (Handley et al., 1992; Bedard-Haughn et al., 2003). Various sources and pools of N$_2$O (soil, oceanic, tropospheric and stratospheric) therefore show distinct O isotopic signatures. Based on this natural abundance variation, the $\delta^{18}$O-N$_2$O signature is increasingly used in addition to $\delta^{15}$N-N$_2$O to characterize the source, production and consumption of N$_2$O (Wahlen et al., 1985; Kim et al., 1990; Yoshinari et al., 1997; Schmidt et al., 2004a; Van Groenigen et al., 2005a; Pérez et al., 2006).

Besides natural abundance methods, combinations of NH$_4^+$ and NO$_3^-$ that are artificially enriched with $^{15}$N are routinely used to study the processes of nitrification and denitrification and their relative contribution to N$_2$O production (Stevens et al., 1997; Panek et al., 2000; Baggs et al., 2003; Bateman et al., 2005). The additional use of $^{18}$O-enriched H$_2$O has been suggested to enable the distinction between N$_2$O from nitrification and nitrifier denitrification (the reduction of NO$_2^-$ to N$_2$O by nitrifiers) (Wrage et al., 2005). Similar to natural abundance studies, in this approach by Wrage et al. (2005) the different contributions of O$_2$-O and H$_2$O-O to N$_2$O-O in the various pathways is used for
Although the exact pathway of N\textsubscript{2}O formation from nitrification is unknown, the O isotopic signature of nitrification-N\textsubscript{2}O is assumed to be identical to that of hydroxylamine (NH\textsubscript{2}OH) and 100% determined by O\textsubscript{2}-O (Wrage et al., 2005). For nitrifier denitrification, the O isotopic signature of N\textsubscript{2}O will equal that of NO\textsubscript{2}-, with half of the O derived from O\textsubscript{2} and half of H\textsubscript{2}O. The O isotopic signature of denitrification-N\textsubscript{2}O will be identical to that of NO\textsubscript{3}- for denitrification of nitrification-derived NO\textsubscript{3}-, 1/3\textsuperscript{rd} of the O will be from O\textsubscript{2} and 2/3\textsuperscript{rd} from H\textsubscript{2}O-O. Denitrification of fertilizer-NO\textsubscript{3}- will produce N\textsubscript{2}O with an O isotopic signature determined by that of the fertilizer (Figure 2.2). These differences in the O origin and the resulting isotopic composition of N\textsubscript{2}O from the various pathways are then used to distinguish between their relative contributions.

One complication in the interpretation of δ\textsuperscript{18}O values of N\textsubscript{2}O is that the exact pathway resulting in N\textsubscript{2}O as a byproduct of nitrification (Figure 2.2) is still
unknown. It is thought to be formed as by-product of incomplete oxidation of NH$_2$OH to NO$_2^-$ (Arp et al., 2003; Stein et al., 2003). If the N$_2$O is produced before the incorporation of the second O atom, the O isotopic signature of nitrification-N$_2$O will indeed be identical to that of NH$_2$OH, and thereby of O$_2$. However, if the second O atom has already been incorporated before the release of N$_2$O, the O isotopic signature of the N$_2$O would also be partly determined by that of H$_2$O. This uncertainty could form a source of error for approaches that assume the O in nitrification-N$_2$O to be 100% derived from O$_2$.

Another complication in the interpretation of $\delta^{18}$O signatures of N$_2$O and NO$_3^-$ is the assumption that no significant O exchange will take place between H$_2$O and other compounds involved in NO$_3^-$ or N$_2$O formation (either sources, intermediates or end-products). Such exchange could be a mechanical process, physically exchanging the O of H$_2$O with that of another compound. It could also occur during reactions that involve the incorporation of O from water, or the release of O to water, when such a reaction is reversible. In that case, alternating occurrence of the forward and reverse reaction would induce O exchange between H$_2$O and the nitrogenous oxides involved in the reaction. If such an exchange would take place at significant rates, the differences in O isotopic signatures of the respective compounds, on which both natural abundance and artificially enriched studies are based, would blur. This would complicate the interpretation of the $\delta^{18}$O-N$_2$O values, possibly leading to incorrect conclusions about the origin of NO$_3^-$ or N$_2$O. The possibility of such an exchange is occasionally mentioned in source determination studies, and in those cases is assumed to be of minor importance under the specific experimental conditions (Wahlen et al., 1985; Toyoda et al., 2005; Wrage et al., 2005; Menyailo et al., 2006a). However, the available literature on O exchange is extensive but largely confined to chemical and microbiological studies on monocultures rather than soils. Its potential impact was mentioned in a few natural abundance studies on marine ecosystems (Casciotti et al., 2002; Sigman et al., 2005), but overall there have been few efforts to evaluate its relevance to source determination studies in terrestrial ecosystems.

With this review paper, we aim to provide a better understanding of the processes of O exchange and their implications for source determination studies. We first summarize the literature on O exchange. We then discuss processes
likely to cause such exchange, and speculate on factors controlling the extent of O exchange in natural ecosystems. Finally, we identify the implications of O exchange for source determination studies of NO₃⁻ and N₂O, and discuss research needs and possibilities to be addressed in the future.

Oxygen exchange reported in literature

Oxygen exchange has been reported to occur through both chemical and biochemical processes. The following text presents the current level of understanding on both.

Chemical oxygen exchange

Table 2.1 lists studies on chemical O exchange between H₂O and various nitrogenous oxides. The exact reactions are unclear, but most studies report that the rate of O exchange was affected by pH and nitrous acid (HNO₂) concentrations. Acidic conditions promote O exchange, and both first and second order rate laws are reported (for [H⁺] as well as [HNO₂]). Bonner and Jordan (1973) showed the rate of O exchange to decrease with increasing NO₂⁻ concentrations. Furthermore, a catalytic effect of chloride ions was found by Anbar and Guttmann (1961).

Chemical oxygen exchange was in these studies investigated in aqueous solutions, often under conditions that are not commonly encountered in

Table 2.1: Studies reporting on chemical O exchange between H₂O and nitrogen oxides.

<table>
<thead>
<tr>
<th>Ion or compound</th>
<th>Exchange effected by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNO₃</td>
<td>Chloride</td>
<td>Anbar et al. (1961)</td>
</tr>
<tr>
<td>NO</td>
<td>pH, nitrous acid; no exchange in pure water</td>
<td>Bonner (1970)</td>
</tr>
<tr>
<td>NO</td>
<td>pH, nitrite &amp; nitrous acid</td>
<td>Bonner et al. (1963)</td>
</tr>
<tr>
<td>HNO₂</td>
<td>pH</td>
<td>Bothner-By et al. (1952)</td>
</tr>
<tr>
<td>HNO₂</td>
<td>pH, nitrous acid</td>
<td>Bunton et al. (1959)</td>
</tr>
<tr>
<td>HNO₂</td>
<td>pH, nitrous acid</td>
<td>Bunton et al. (1959)</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>pH</td>
<td>Van Etten et al. (1981)</td>
</tr>
<tr>
<td>HNO₂</td>
<td>pH, nitrite / nitrous acid, hydrogen peroxide</td>
<td>Anbar et al. (1954)</td>
</tr>
<tr>
<td>KNO₂, KNO₃</td>
<td>No oxygen exchange, no effect of pH</td>
<td>Hall et al. (1940)</td>
</tr>
</tbody>
</table>
Oxygen exchange between (de)nitrification intermediates and H₂O: a review

ecosystems. Solutions were sometimes highly acidic (e.g. pH<1 in Bunton and Stedman (1959a)), and temperatures frequently higher or lower than they would normally be in most soils. Temperatures were 0°C or lower in Bothner-By and Friedman (1952), Bunton et al. (1959b), and Bunton and Stedman (1959a); around 25°C in Anbar and Taube (1954), Bonner (1970), Bonner and Jordan (1973) and Van Etten and Risley (1981); and 60 to 100°C in Anbar and Guttmann (1961). Also, substrate concentrations were often much higher than in terrestrial ecosystems. For example, NO₂⁻ and HNO₂ concentrations were up to 3 M and 1.13 M, respectively, in Bunton et al. (1959b).

Little is known about chemical exchange at near-neutral pH. Some indication may be derived from Casciotti et al. (2007) who evaluated the effect of storage conditions on the O isotopic signature of NO₂⁻ in freshwater and seawater samples at various pH values, temperatures and NO₂⁻ concentrations. In particular at pH 6 and 8 at 4°C, they found substantial exchange of O between NO₂⁻ and H₂O, totaling 10-30% within 3 weeks of storage. This may indicate a potential for O exchange in soil moisture under normal conditions. However, it is doubtful whether the exchange observed was strictly chemical since the samples analyzed were derived from freshwater and marine ecosystems. Microorganisms present in the samples may likely be responsible for the observed exchange.

Based on our examination of the current literature we conclude that the possibility for chemical O exchange in soils can not be excluded. However, we consider chemical O exchange unlikely to be significant in soils, as there is currently no proof of its occurrence or extent under conditions that normally prevail in soil ecosystems.

Biochemical oxygen exchange

While proof is lacking on the occurrence and significance of chemical O exchange in soil ecosystems, this is not the case when considering biochemical exchange. The remainder of this study we focus on biochemical exchange. The processes during which biochemical O exchange is reported to occur are all enzymatically catalyzed. We will discuss these processes, their thermodynamics and the enzymes involved. Finally, we review ecosystem studies that may indirectly provide support for the occurrence of substantial O exchange in soil ecosystems.
**Nitrification pathway**

Biochemical O exchange has been associated with several nitrifiers (Table 2.2), including both ammonia (NH₃) and nitrite oxidizers. Andersson et al. (1982) analyzed the δ¹⁸O of NO₂⁻ derived from NH₄⁺ and hydroxylamine (NH₂OH) oxidation by the NH₃ oxidizer *Nitrosomonas europaea* in the presence of ¹⁸O enriched H₂O. They reported O exchange between NO₂⁻ and H₂O. The NO₂⁻ oxidizer *Nitrobacter agilis* grown on ¹⁵N¹⁶O₂⁻ in the presence of ¹⁸O enriched H₂O produced zero, single and double ¹⁸O labeled NO₃⁻ (Kumar et al., 1983). This double ¹⁸O labeled NO₃⁻ could only have been formed as a result of O exchange during NO₂⁻ oxidation to NO₃⁻ (Kumar et al., 1983). DiSpirito et al. (1986) reported O exchange between NO₃⁻ molecules was catalyzed by *Nitrobacter winogradskyi* grown on NO₂⁻. Albeit to a smaller extent than between NO₃⁻ molecules, O exchange also occurred between H₂O and NO₃⁻ (DiSpirito et al., 1986).

Ammonia oxidation takes place in two steps: first NH₃ is oxidized to NH₂OH, which is then oxidized to NO₂⁻. The former process is catalyzed by the membrane bound enzyme ammonia mono-oxygenase, and requires O₂ and the input of electrons (Arp et al., 2002; Arp et al., 2003; Fiencke et al., 2006) (Figure 2.3). The required electrons are provided by the second step, the conversion of NH₂OH to NO₂⁻, which is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO) that is located in the periplasm (Arp et al., 2002; Arp et al., 2003) (Figure 2.3). This conversion yields additional reducing equivalents that are needed to gain energy from this reaction. This is an important point with respect to the possibility of O exchange; as this second step involves the incorporation of O from H₂O (Figure 2.4(a)), the reverse of the process could thus allow the exchange of O. However, NH₃ oxidizers are mainly obligatory lithoautotrophic organisms and gain energy

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>Measured Ion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitrosomonas europaea</em></td>
<td>NH₄⁺, NH₂OH</td>
<td>NO₂⁻, NO₃⁻</td>
<td>Andersson et al. (1982)</td>
</tr>
<tr>
<td><em>Nitrobacter agilis</em></td>
<td>NO₂⁻</td>
<td>NO₃⁻</td>
<td>Kumar et al. (1983)</td>
</tr>
<tr>
<td><em>Nitrobacter winogradskyi</em></td>
<td>NO₂⁻</td>
<td>NO₃⁻</td>
<td>DiSpirito et al. (1986)</td>
</tr>
</tbody>
</table>
from NH₃ oxidation for their growth. The conversion of NH₂OH to NO₂⁻ is essential to provide electrons for NH₃ oxidation to NH₂OH. It would thus be unbeneﬁcial and therefore unlikely that the reverse of the reaction of NH₂OH oxidation to NO₂⁻ would take place and allow for O exchange by NH₃ oxidizing nitrifiers. In addition, *Nitrosomonas europaea* is the only NH₃ oxidizer studied with regard to O exchange (Andersson et al., 1982), but it is not a very common nitrifier in the soil. It is rather unrepresentative of this group of nitrifiers in a variety of traits (Casciotti et al., 2001; Kowalchuk et al., 2001; Arp et al., 2003; Wrage et al., 2004b; Shaw et al., 2006), and this possibly extends to its behavior regarding O exchange. Recently, it has been reported that Archaea may also be capable of NH₃ oxidation (Könneke et al., 2005), and that such Archaea constitute a signiﬁcant part of the NH₃ oxidizing community in soil (Leininger et al., 2006). However, little is known about (dis-)similarities between these Archaea and the better-known bacterial NH₃ oxidizers. Altogether, we can only speculate about O exchange induced by NH₃ oxidation in soil systems.

In nitrification, NH₃ oxidation is followed by the oxidation of NO₂⁻ to NO₃⁻. This process is catalyzed by the membrane-bound enzyme nitrite-oxidoreductase (Bock et al., 1986). Again, the reversibility of this step could allow for the exchange of O atoms between H₂O and NO₂⁻ or NO₃⁻ (Figure 2.3(b)) (Aleem, 1968; Sundermeyer-Klinger et al., 1984; Wood, 1986). Removal of reducing equivalents
(H₂), by burning with O₂, is needed to pull this reaction forward (towards production of NO₃⁻). Because of these thermodynamics of the reaction, the reaction is probably close to equilibrium and the reverse may occur as well, thereby allowing for O exchange.

In addition it should be noted that the two NO₂⁻ oxidizing nitrifiers studied and reported to catalyze O exchange between NO₂⁻ and H₂O are of the genus Nitrobacter (Kumar et al., 1983; DiSpirito et al., 1986). These form an exceptional group of nitrifiers in that they are not strictly autotrophic and aerobic. Nitrobacter can grow heterotrophically (Bock et al., 1986) while repressing the nitrite-oxidizing system (Steinmüller et al., 1977), and they are also capable of anaerobic growth, converting NO₃⁻ to NO₂⁻ with pyruvate, acetate or glycerol as electron donors (Aleem et al., 1981; Sundermeyer-Klinger et al., 1984). Compared to strictly autotrophic NO₂⁻ oxidizers, these organisms are thus less dependent on the energy derived from NO₂⁻ oxidation. The argument that the reverse reaction would not take place because it is energetically unfavorable may therefore not hold for these NO₂⁻ oxidizers. The reverse reaction, and thereby O exchange, is likely to occur in this case. For strictly autotrophic NO₂⁻ oxidizers, the occurrence and extent of the reverse reaction remains speculative.

Denitrification pathway

Oxygen exchange between H₂O and nitrogen oxides (NO₃⁻, NO₂⁻ and nitric oxide (NO)) has been quantified for a number of denitrifiers, and was observed to take place at least to some extent in all denitrifiers studied (Table 2.3) (Garber et al., 1982; Aerssens et al., 1986; Shearer et al., 1988; Ye et al., 1991; Casciotti et al., 2002). The denitrifiers studied were all bacteria, so the discussion below will
consider the processes and enzymes involved in bacterial denitrification only (as opposed to fungal denitrification).

The O exchange is quantified by measuring the incorporation of O from artificially ¹⁸O enriched H₂O into either N₂O or NO₂⁻. The extent of O exchange differed both between species and substrates (Table 2.3). Casciotti et al. (2002) found O exchange during NO₃⁻ reduction to N₂O to be small for *Pseudomonas aureofaciens* (< 10% incorporation of H₂O-O into N₂O), but approximately 30% for *Corynebacterium nephridii* and up to 78% for *Pseudomonas chlororaphis*. Ye et al. (1991) studied O exchange during NO₃⁻ and NO reduction to N₂O for eight

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>Measured ion or compound</th>
<th>Percentage of exchange (d)</th>
<th>NiR type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paracoccus denitrificans</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>59 (heme-cd1)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>76 (heme-cd1)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>58 (heme-cd1)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>39 (heme-cd1)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>94 (copper)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Achromobacter cycloclastes</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>4 (copper)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aureofaciens</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>6 (copper)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodopseudomonas sphaeroides</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>90 (copper)</td>
<td></td>
<td>Ye et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Paracoccus denitrificans</em></td>
<td>NO⁻</td>
<td>NO³⁻; N₂O</td>
<td>32; 100 (heme-cd1)</td>
<td>heme-cd1</td>
<td>Garber &amp; Hollocher (1982)</td>
</tr>
<tr>
<td><em>Pseudomonas denitrificans</em></td>
<td>NO⁻</td>
<td>NO³⁻; N₂O</td>
<td>12; 70 (heme-cd1)</td>
<td>heme-cd1</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>NO⁻</td>
<td>N₂O</td>
<td>8 - 35 (heme-cd1)</td>
<td></td>
<td>Aerssens et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>N₂O</td>
<td>13 - 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>NO⁻</td>
<td>NO³⁻</td>
<td>5 - 8 (heme-cd1)</td>
<td></td>
<td>Shearer &amp; Kohl (1988)</td>
</tr>
<tr>
<td></td>
<td>NO⁰</td>
<td>NO₃⁻</td>
<td>&lt; 10</td>
<td>copper</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NO⁰</td>
<td>NO₃⁻</td>
<td>30</td>
<td>copper</td>
<td>Casciotti et al. (2002)</td>
</tr>
</tbody>
</table>

In the end of incubation; total incubation period differs for different experiments.
denitrifiers. They observed exchange rates up to 94% during NO$_2^-$ reduction. During reduction of NO, the amount of O incorporated from H$_2$O into N$_2$O ranged between 4 and 84%. No O from H$_2$O was incorporated in N$_2$O when pure cultures of *Pseudomonas aureofaciens* were incubated with N$_2$O, showing that O exchange takes place during reduction of NO$_2^-$ and/or NO to N$_2$O, and not with N$_2$O itself (Ye et al., 1991). Aerssens et al. (1986) also determined O exchange during both NO$_2^-$ and NO reduction, by *Pseudomonas stutzeri*. They report the extent of O exchange between H$_2$O and the produced N$_2$O to range between 8 and 35% for NO$_2^-$ reduction, and between 13 and 31% for NO reduction. In addition, they found O exchange to decrease with increasing NO$_2^-$ concentrations. Shearer and Kohl (1988) reported the incorporation of O from $^{18}$O-labeled H$_2$O in NO$_2^-$ by *Pseudomonas stutzeri* to range between 5 and 8%. Garber and Hollocher (1982) found the extent of exchange to differ among denitrifiers, but to be present in all species studied.

Most denitrifier studies have concentrated on O exchange during NO$_2^-$ and NO reduction to N$_2$O. The NO$_2^-$ reduction to NO in the denitrification pathway is an enzyme-bound dehydration step including the incorporation of H$^+$ and formation of H$_2$O (Figure 2.5). This step is reversible and hence allows for O exchange (Averill et al., 1982; Garber et al., 1982; Kim et al., 1984; Weeg-Aerssens et al., 1987; Shearer et al., 1988; Weeg-Aerssens et al., 1988). Enzymes responsible for NO$_2^-$ reduction to NO are of two distinct types: cytochrome cd$_1$ and copper-containing nitrite reductase (heme-cd$_1$-NiR and copper-NiR, respectively) (Hochstein et al., 1988; Averill, 1996). Kim and Hollocher (1984) have shown that NiRs of the heme-cd$_1$ type are able to catalyze NO$_2^-$/H$_2$O-O exchange. Ye et al. (1994) also found significant rates of O exchange during NO$_2^-$ reduction for all four heme-cd$_1$-NiR containing denitrifiers studied. They showed O exchange to be possible in copper-NiR containing species as well, but the extent differed considerably between the four species studied (Table 2.3). The latter may be explained by the fact that copper-NiR containing organisms exhibit quite extreme physiological diversity (Coyne et al., 1989; Ye et al., 1991; Averill, 1996). Diversity in functional enzymes may thus result in differences in the extent of exchange. In nature, the heme-cd$_1$-NiR is present in about two-third of the denitrifying species examined (Hochstein et al., 1988; Averill, 1996). Overall, organisms with the ability to catalyze O exchange during NO$_2^-$ reduction are very likely to be present...
Oxygen exchange between (de)nitrification intermediates and H$_2$O: a review

For NO reduction, three types of nitric oxide reductase (NOR) have been identified in bacteria: cNOR, qNOR and qCuNOR (Tavares et al., 2006). All three are membrane-bound enzymes. Although structure and composition of their inactive subunits differ, their active site structure is thought to be highly homologous (Tavares et al., 2006). While theoretical studies have pointed out the nature of the enzymatic reaction of NO reduction, the exact mechanism is still unclear (Tavares et al., 2006). With respect to O exchange, Ye et al. (1991) reported differences in the rate of exchange during NO reduction for species differing in their NiR-type. Significantly higher extents of exchange were found for the copper-NiR than the heme-cd$_1$-NiR containing species (Table 2.3). It may be hypothesized that species with distinct NiRs also have different NORs, causing differences in exchange during NO reduction.

Although not as intensively studied as NO$_2^-$ and NO reduction, the possibility of O exchange during the first step of denitrification, the reduction of NO$_3^-$ to NO$_2^-$, should also be considered. Exchange of O during denitrification of NO$_3^-$ to N$_2$O has been reported (Casciotti et al., 2002), but the results could be interpreted as a result of O exchange during later stages of the denitrification process. Nitrate reductases appear to be fairly similar among denitrifying bacteria (Averill, 1996). Respiratory NO$_3^-$ reduction involves the nitrate reductase NaR, which is located in the cytoplasmic membrane, with its active site in the cytoplasm (Ye et al., 1994; Averill, 1996; Tavares et al., 2006; Wallenstein et al., 2006). In contrast, NiR is a soluble enzyme in the periplasmic space and NOR is bound to the cytoplasmic membrane, but has its active site in the periplasm (Figure 2.6). For NO$_3^-$ reduction to NO$_2^-$, NO$_3^-$ thus first has to pass the cytoplasmic membrane, and NO$_2^-$ needs to be transported back into the periplasm. So, for O exchange with ($^{18}$O$^{-}$) H$_2$O to take place, the H$_2$O would need to pass the membrane as well.

\[
\text{NO}_2^- + E \leftrightarrow \text{E} \cdot \text{NO}_2^-
\]

\[
\text{E} \cdot \text{NO}_2^- + 2H^+ \leftrightarrow \text{E} \cdot \text{NO}^+ + H_2O
\]

Figure 2.5: The step of NO$_3^-$ reduction in denitrification to N$_2$O and N$_2$ is an enzyme-bound, reversible dehydration step (E = enzyme) (Averill et al., 1982; Garber et al., 1982; Kim et al., 1984; Weeg-Aerssens et al., 1987 and 1988; Shearer et al., 1988).
while this is not needed for O exchange during NO\textsubscript{2} and NO reduction. The exchange of O may thus be less likely during the first part of the bacterial denitrification pathway. A second type of nitrate reductase is known, NaS, which participates in nitrogen assimilation (Lin et al., 1998; Richardson et al., 2001). This enzyme is located in the cytoplasmic space (Lin et al., 1998; Richardson et al., 2001) (as is the active site of NaR), so the H\textsubscript{2}O would also need to pass the cytoplasmic membrane for O exchange during NO\textsubscript{3} reduction to NO\textsubscript{2} (as part of N assimilation) catalyzed by this enzyme. Yet another respiratory nitrate reductase is known which is involved in nitrate respiration that is coupled to quinol oxidation (Richardson et al., 2001; Tavares et al., 2006). This NaP is located in the periplasmic space (Berks et al., 1994; Berks et al., 1995a; Berks et al., 1995b; Richardson et al., 2001; Tavares et al., 2006). So for O exchange with H\textsubscript{2}O to take place upon NO\textsubscript{3} reduction by microorganisms that possess this enzyme, the H\textsubscript{2}O (and NO\textsubscript{3}) does not need to pass the cytoplasmic membrane.

The last compound left in the denitrification pathway that may be subject to O exchange is N\textsubscript{2}O. As N\textsubscript{2}O formation is chemically (Bonner et al., 1952) and enzymatically (St. John et al., 1977) irreversible, O exchange between H\textsubscript{2}O and N\textsubscript{2}O is very unlikely. Results of Ye et al. (1991) also confirm this, since no \textsuperscript{18}O from \textsuperscript{18}O-H\textsubscript{2}O was incorporated into N\textsubscript{2}O when pure cultures of Pseudomonas aureofaciens were incubated with N\textsubscript{2}O.

To summarize, O exchange during the process of denitrification seems to be

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**Figure 2.6: Arrangement of the enzymes involved in denitrification in Gram-negative bacteria.** NaR = nitrate reductase; NiR = nitrite reductase; NoR = nitric oxide reductase; NoS = nitrous oxide reductase. Modified from Ye et al. (1994), Averill (1996) and Wallenstein (2006).
mainly associated with NO$_2^-$ and NO reduction. All the reactions in the stepwise reduction of NO$_3^-$ to N$_2$ are exergonic (Averill, 1996), but the energy gain is smallest for NO$_2^-$ reduction. Reduction of NO$_2^-$ could therefore be argued to be the major contributor to O exchange since this step is most likely to be reversed. Garber and Hollocher (1982) even argued, based on required functional traits and kinetic considerations, that NiR is the only reasonable candidate as the enzyme responsible for the catalysis of O exchange during denitrification.

**Nitrifier denitrification pathway**

The only organism studied with respect to O exchange that is capable of nitrifier denitrification is *Nitrosomonas europaea*. However, O exchange in this microorganism was studied with respect to the nitrification pathway (Andersson et al., 1982). As *N. europaea* is not very common in the soil and because N$_2$O production through the nitrifier denitrification pathway by this organism differs from that in other nitrifiers (Casciotti et al., 2001; Kowalchuk et al., 2001; Arp et al., 2003; Wrage et al., 2004b; Shaw et al., 2006), there is effectively no literature on O exchange during the nitrifier denitrification pathway in soil. Current understanding on the enzymes involved in this pathway may provide some insight. The enzyme responsible for NO$_2^-$ reduction by nitrifiers has been identified as a copper-containing NiR that seems very similar to that found in denitrifiers (Casciotti et al., 2001; Chain et al., 2003; Cantera et al., 2007), possibly as a result of lateral gene transfer (Garbeva et al., 2007). It could thus be hypothesized to behave similarly to copper-NiR in denitrifiers. However, the behavior of copper-NiR containing denitrifiers with respect to O exchange was very diverse (Table 2.3), which suggests that the extent of O exchange during nitrifier denitrification could be similarly diverse. Literature suggests that the NOR in denitrifying ammonia oxidizers may be similar to that in denitrifiers as well. Casciotti and Ward (Casciotti et al., 2005) identified the widespread occurrence of genes encoding NOR in strains of ammonia oxidizing bacteria, including *Nitrosomonas* and *Nitrosococcus* spp., similar to norB gene sequences from denitrifiers. Garbeva et al. (Garbeva et al., 2007) found gene sequences of norB (and nirK) from several *Nitrospira* spp. not phylogenetically different from those of denitrifiers. If the NOR in ammonia oxidizers is indeed similar to that in denitrifiers, nitrifiers capable of carrying out the nitrifier denitrification pathway
will be also able to catalyze O exchange with NO as substrate. Although future research needs to explore this further, we can hypothesize that O exchange may occur during nitrifier denitrification.

**Soil studies**

The studies summarized above show that O exchange has been reported in many major groups of nitrifiers and denitrifiers. Temperatures and pH in these experiments were not unrealistic for soil. This suggests that O exchange catalyzed by such microorganisms can be expected in soil as well. However, these were all studies on monocultures incubated in a nutrient medium. The importance of O exchange in functioning soil ecosystems has not been established yet.

Several soil incubation studies indirectly suggest the occurrence of O exchange. Wrage et al. (2005) incubated soil with $^{18}$O enriched H$_2$O at 1.0 atom% excess. In the presence of acetylene (C$_2$H$_2$; inhibiting nitrification and the reduction of N$_2$O to N$_2$), the δ$^{18}$O values of N$_2$O did not increase above background levels (Figure 2.7). Soil was also incubated without C$_2$H$_2$ to determine N$_2$O production and its O isotopic signature. In the absence of O exchange, the maximum $^{18}$O enrichment that could be reached in the produced N$_2$O would then be 67% of the atom% in H$_2$O (Figure 2.2); or 0.67 atom% excess in this case. This theoretical maximum could only be reached if all N$_2$O would be produced through nitrification-coupled denitrification of the applied NH$_4^+$, without any N$_2$O resulting from nitrification, nitrifier denitrification or denitrification of applied NO$_3^-$ (Figure 2.2). This was unlikely as considerable amounts of NH$_4^+$ and NO$_3^-$ had been applied to the soil. However, within 24 hours the O isotopic signature of N$_2$O produced became identical to that of the $^{18}$O enriched H$_2$O (Figure 2.7, Wrage et al. (2005)). A reinterpretation of these results therefore seems to suggest (i) extensive and rapid O exchange between H$_2$O and intermediate compounds of the N$_2$O producing pathway(s); and (ii) a biochemical rather than a chemical controlled exchange process, as this exchange is inhibited by C$_2$H$_2$.

Menyailo and Hungate (2006a) applied $^{18}$O enriched NO$_3^-$ to forest soils and measured the δ$^{18}$O of the produced N$_2$O. Under circumstances where both nitrification and the reduction of N$_2$O to N$_2$ were inhibited, the O isotopic signature of N$_2$O produced should be identical to that of NO$_3^-$ present in the soil.
However, the maximum $^{18}$O-N$_2$O was 1.13 atom% excess, while that of the NO$_3^-$ applied was 1.40 atom% excess. Incomplete inhibition of nitrification and the reduction of N$_2$O to N$_2$ should be considered as possible explanations for this lower enrichment. However, O exchange between the enriched NO$_3^-$ and unlabeled H$_2$O could be an alternative explanation.

**Discussion - Interference with stable isotope tracing studies**

To our knowledge, O exchange between H$_2$O and nitrogenous oxides during nitrification and denitrification has not been quantified in terrestrial ecosystems, neither in field studies nor in laboratory incubations. As O isotopic analyses are now increasingly used to study N turnover processes in these ecosystems, information about the occurrence of O exchange is crucial to evaluate the reliability of these studies. Below, we discuss the possible implications of the process of O exchange for such studies.

**Source determination of NO$_3^-$**

Biological processes have a significant effect on the natural abundance isotopic composition of NO$_3^-$. Analyses of $\delta^{15}$N- and $\delta^{18}$O-NO$_3^-$ are commonly used to discriminate between NO$_3^-$ sources and to evaluate the residence time of NO$_3^-$ in
Chapter 2

the soil-plant environment (Amberger et al., 1987; Durka et al., 1994; Mengis et al., 2001; Williard et al., 2001; Burns et al., 2002; Pardo et al., 2004). In many of these studies the δ^{18}O of the NO\textsubscript{3} is low compared to atmospheric NO\textsubscript{3} (+15 to +75‰ SMOW), but closer to the range expected from nitrification of soil N (-5 to +15‰ SMOW) (Figure 2.1). It is consequently reasoned that most NO\textsubscript{3} in e.g. groundwater or drainage water is derived from microbial nitrification of soil-N within the soil system, with little direct contribution of atmospheric deposition. However, soil water has an even lower δ^{18}O (about -25 to +4‰ SMOW, Figure 2.1). Therefore, exchange of O atoms between NO\textsubscript{3} and H\textsubscript{2}O (as a result of reversible processes of NO\textsubscript{3} transformation where O is incorporated from or released to H\textsubscript{2}O) would decrease the δ^{18}O signature of the NO\textsubscript{3}. Depending on the extent of this exchange, the resulting net δ^{18}O-NO\textsubscript{3} could be any intermediate between the δ^{18}O of H\textsubscript{2}O and that of the actual source(s). The contribution of nitrification of soil N to the NO\textsubscript{3} pool could therefore be overestimated if O exchange is not taken into account. However, at equilibrium NO\textsubscript{2} and NO\textsubscript{3} appear to be slightly enriched in 18O compared to water (equilibrium isotope effect) (Bohlke et al., 2003; Casciotti et al., 2007). Reliable quantitative knowledge on this effect remains unavailable as only these two studies have addressed this issue.

Analyses of δ^{18}O- (and δ^{15}N-) NO\textsubscript{3} are also used to evaluate the progress of denitrification (Böttcher et al., 1990; Wassenaar, 1995; Aravena et al., 1998; Mengis et al., 1999; Mengis et al., 2001; Groffman et al., 2006; Panno et al., 2006; Sebilo et al., 2006). During denitrification, isotope fractionation leaves the residual NO\textsubscript{3} relatively enriched in the heavier isotopes 18O and 15N, which is used to assess the role of denitrification. However, if O exchange with H\textsubscript{2}O takes place, the δ^{18}O-NO\textsubscript{3} would decrease again, leading to a possible underestimation of the rate of denitrification.

Source determination of N\textsubscript{2}O

Measurements of δ^{18}O and δ^{15}N of N\textsubscript{2}O are used to study the production and consumption of N\textsubscript{2}O, and to distinguish between pools of N\textsubscript{2}O (Wahlen et al., 1985; Kim et al., 1990; Kim et al., 1993; Yoshinari et al., 1997; Tilsner et al., 2003; Schmidt et al., 2004a; Wrage et al., 2004c; Van Groenigen et al., 2005a; Pérez et al., 2006). For such studies, the occurrence of O exchange upon processes of N\textsubscript{2}O formation and consumption may cause similar problems as for NO\textsubscript{3} source
determination. Interpretation of the isotopic signatures for source determination is based on distinct isotopic signatures from different N₂O pools (soil, oceans, stratosphere), and isotopic fractionation is again used to evaluate the process of denitrification to N₂O and further reduction (consumption) to N₂ in soil. In this process of N₂O reduction, isotope fractionation causes a relative enrichment of the heavier isotopes in N₂O. However, O exchange during reduction of NO₃⁻ and/or NO₂⁻ to N₂O with H₂O would dilute the O pool of these nitrogenous oxides with ¹₆O, and lead to a lower δ¹⁸O-N₂O signature. If such an exchange effect is not accounted for, this would lead to incorrect interpretation of the δ¹⁸O-N₂O signature, and to an underestimation of the contribution of denitrification.

In the study of Wrage et al. (2005), soil treated with ¹⁸O enriched H₂O in the presence of C₂H₂ produced N₂O of which the O isotopic signature did not differ from background levels, suggesting the absence of O exchange. However, the data of the non-acetylene incubations indicated the presence of biochemical O exchange; the O isotopic signature of the N₂O produced often reached values close to that of the ¹⁸O enriched H₂O of the treatment. This methodology aims at distinguishing N₂O production from nitrification, nitrifier denitrification and denitrification. O exchange interferes with such experiments since it increases the ¹⁸O value of the N₂O. It thereby leads to an overestimation of the contribution of nitrifier denitrification and nitrification-coupled denitrification to total N₂O production.

To summarize, the presence of O exchange between H₂O and nitrogenous oxides and the uncertainty about its extent is relevant to the interpretation of ¹⁸O data from studies on (i) source determination of NO₃⁻; (ii) evaluation of soil processes and residence time of NO₃⁻ and soil-N in general (cycling through biota and nitrification); (iii) estimates on the progress of denitrification; (iv) source determination of N₂O; and (v) distinction between pathways of N₂O production. Considering previous studies on O exchange and plausible processes that facilitate such exchange, major concerns are especially associated with N₂O source determination studies. Published literature mainly emphasizes O exchange with intermediates (NO₂⁻ and NO) of denitrification. Therefore, the O isotopic signature of N₂O is likely to be more affected by O exchange than that of NO₃⁻ and its ¹⁸O analysis thus more susceptible to misinterpretation.
Discussion - Research recommendations

As discussed above, the presence of O exchange can interfere with NO$_3^-$ and N$_2$O source determination studies. To enable correct interpretation of O isotopic signatures in such studies, quantitative knowledge on O exchange in soil is required. We suggest that future research should, next to extending monoculture studies, focus on developing methods to quantify O exchange in soil. Below, we will discuss some challenges and possible experimental approaches.

At natural abundance levels it is impossible to make a reliable distinction between $^{18}$O fractionation and $^{18}$O exchange. Therefore, we postulate that quantification of O exchange needs to be done with artificially enriched $^{18}$O compounds. The use of $^{18}$O enriched H$_2$O in combination with O isotopic analyses of NO$_3^-$ and N$_2$O could provide such information.

Measuring the incorporation of $^{18}$O from labeled H$_2$O into N$_2$O may already identify the presence of O exchange. However, since usually the relative contributions of nitrification, denitrification and nitrifier denitrification to N$_2$O production are unknown, this does not allow quantification of the extent of O exchange. We propose that research should therefore first focus on the few soil conditions where the contribution of these processes is known. The best opportunity for this is soil where denitrification is the sole process of N$_2$O production. This could be reliably checked using combinations of $^{15}$N enriched NH$_4^+$ and NO$_3^-$ with subsequent $^{15}$N-N$_2$O analyses. If in such a system $^{18}$O enriched H$_2$O is applied, the $^{18}$O signal of the produced N$_2$O would be a direct quantification of O exchange during denitrification.

In addition to $^{18}$O enriched H$_2$O, the use of $^{18}$O enriched NO$_3^-$ is an especially promising tool for studying O exchange. Combinations of treatments with $^{15}$N and $^{18}$O enriched NO$_3^-$ and subsequent isotopic analyses of N$_2$O would allow the quantification of O exchange during denitrification. Without O exchange, the ratio of the N and O enrichment in the produced N$_2$O should equal that of the applied NO$_3^-$. In theory, this would hold in any soil system, regardless of the relative contribution of denitrification to N$_2$O production.

It is clear that $^{18}$O analyses of NO$_3^-$ would provide another useful tool in studying O exchange. Determination of the $^{18}$O signature of soil NO$_3^-$ may be done on soil extracts. However, such soil extraction for mineral N analyses is
often done with KCl at relatively high concentrations (1-2 M). Although it is unclear whether significant chemical exchange may take place in soil, chloride ions are reported to catalyze chemical O exchange in aqueous solutions (Anbar et al., 1961). The possibility of the occurrence of O exchange in such soil KCl extraction solutions, prior to O isotopic analyses, should therefore be considered.

Similar to all the other biochemical processes involved, the extent of O exchange is likely to be dependent on soil conditions like pH, moisture content and temperature. Consequently, the effect of these parameters on the extent of O exchange needs to be identified. In particular, in order to assess complications relating to source determination using isotope analysis, it needs to be determined whether $^{18}$O exchange is progressive with time or whether it occurs at fixed rates during the process of (de)nitrification.

To summarize, a method needs to be developed to study the extent of O exchange in soil systems, as well as its controlling factors. Such analysis of O exchange should then be included in methodology using $^{18}$O analyses of $N_2O$, and preferably also $NO_3^-$, to correctly interpret the isotopic data.

Conclusion

The literature reviewed demonstrates that most major groups of nitrifiers and denitrifiers are able to catalyze O exchange between $NO_2^-$ and $H_2O$. Oxygen exchange, especially during denitrification, is likely to be significant in most soils, but uncertainty about the extent of exchange and its controls remains and needs to be studied. The occurrence of O exchange is a concern for isotope tracer methods using $^{18}$O analyses, both at natural abundance and artificially enriched levels. Consequently, quantification of this exchange by microbial communities in the soil is necessary. As it is difficult to make a reliable distinction between $^{18}$O fractionation and $^{18}$O exchange at natural abundance levels, this needs to be done with artificially enriched $^{18}$O compounds. In particular, we suggest that future research should focus on developing methods to quantify O exchange in soil (as opposed to in monocultures). In addition, it needs to be determined whether $^{18}$O exchange is progressive with time or occurs at fixed rates (depending on environmental conditions) during the process of (de)nitrification. This is essential for correct interpretation of O isotopic signatures from tracer studies.
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If O exchange in natural systems is significant but not corrected for, $^{18}$O analysis may lead to an overestimation of microbial nitrification of soil N as the source of both $\text{NO}_3^-$ and $\text{N}_2\text{O}$ compared to fertilizer, manure and atmospheric deposition. We conclude that especially the $^{18}$O signature of $\text{N}_2\text{O}$ should only be used with extreme caution in N source determination studies.

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Oxygen exchange between (de)nitrification intermediates and H$_2$O: a review
The $^{18}$O signature of biogenic nitrous oxide is determined by oxygen exchange with water

Abstract  To effectively mitigate emissions of the greenhouse gas nitrous oxide (N$_2$O) it is essential to understand the biochemical pathways by which it is produced. The $^{18}$O signature of N$_2$O is increasingly used to characterize these processes. However, assumptions on the origin of the O atom and resultant isotopic composition of N$_2$O that are based on reaction stoichiometry may be questioned. In particular, deficient knowledge on O exchange between H$_2$O and nitrogen oxides during N$_2$O production complicates the interpretation of the $^{18}$O signature of N$_2$O. Here we studied O exchange during N$_2$O formation in soil, using a novel combination of $^{18}$O and $^{15}$N tracing. Twelve soils were studied, covering soil and land-use variability across Europe. All soils demonstrated the significant presence of O exchange, as incorporation of O from $^{18}$O enriched H$_2$O into N$_2$O exceeded their maxima achievable through reaction stoichiometry. Based on the retention of the enrichment ratio of $^{18}$O and $^{15}$N of NO$_3^-$ into N$_2$O, we quantified O exchange during denitrification. Up to 97% (median 85%) of the N$_2$O-O originated from H$_2$O instead of from the denitrification substrate NO$_3^-$. We conclude that in soil, the main source of atmospheric N$_2$O, the $^{18}$O signature of N$_2$O is mainly determined by H$_2$O due to O exchange between nitrogen oxides and H$_2$O. This challenges the assumption that the O of N$_2$O originates from O$_2$ and NO$_3^-$ as well, in ratios reflecting reaction stoichiometry.

Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). Concerns about rising concentrations of atmospheric N\textsubscript{2}O and the need to develop effective mitigation strategies have led to increased interest in its biochemical production pathways. Oxygen isotopic analyses, generally expressed as its \textsuperscript{18}O signature, are commonly used in NO\textsubscript{3}\textsuperscript{-} source determination and suggested to be a promising tool to study production and consumption of N\textsubscript{2}O as well (Yoshinari et al., 1985; Böttcher et al., 1990; Kim et al., 1990; Durka et al., 1994; Cliff et al., 1997; Yoshinari et al., 1997; Naqvi et al., 1998; Pérez, 2005; Wrage et al., 2005; Menyailo et al., 2006b; Oelmann et al., 2007; Rock et al., 2007). Nitrification, nitrifier denitrification, and denitrification have been identified as the major microbial N\textsubscript{2}O producing pathways in soils and oceans (Firestone et al., 1989; Granli et al., 1994). Based on their reaction stoichiometry, the relative contribution of O\textsubscript{2} and H\textsubscript{2}O as sources of the O in N\textsubscript{2}O differs for these processes (Figure 3.1). The O isotopic composition of N\textsubscript{2}O is therefore considered to be distinct for these different pathways (Pérez, 2005; Wrage et al., 2005). However, the use of oxygen isotopic analyses to characterize these processes might be impaired by O exchange between H\textsubscript{2}O and intermediates (e.g. nitrite, nitrate) in the various N\textsubscript{2}O production pathways, which might alter the O isotopic signature (Kool et al., 2007). (Throughout this thesis, ‘oxygen exchange’ is used as short for the exchange of O between nitrogen oxides and H\textsubscript{2}O.) Oxygen exchange between such intermediates and H\textsubscript{2}O can be catalyzed by a variety of major groups of nitrifiers and denitrifiers (Kool et al., 2007). However, ecosystem studies using isotopes to determine sources of N\textsubscript{2}O rarely consider the possible exchange of O between H\textsubscript{2}O and nitrogen oxides. To our knowledge, its significance has never been established for soils, which constitute the main source of atmospheric N\textsubscript{2}O (IPCC, 2007).

Here we evaluated the significance of O exchange during N\textsubscript{2}O production in soil. We developed novel methodology using \textsuperscript{18}O enriched H\textsubscript{2}O and \textsuperscript{18}O and \textsuperscript{15}N enriched NO\textsubscript{3}\textsuperscript{-}, combined with N\textsubscript{2}O isotopic analyses, to study the process of O exchange. In a series of laboratory incubation experiments on 12 soils covering European soil and land-use variability, we identified the presence and quantified the extent of O exchange in soil.
The \(^{18}\text{O}\) signature of biogenic \(\text{N}_2\text{O}\) is determined by \(\text{O}\) exchange with water.

Figure 3.1: Incorporation of oxygen (O) from \(\text{O}_2\) and \(\text{H}_2\text{O}\) into nitrogen oxides during nitrification, denitrification and nitrifier denitrification, following reaction stoichiometry.

**Methods**

**Soil incubation**

Soil samples were collected from 12 soils across Europe, of which location, soil properties and land-use are summarized in Table 3.1. The upper 10 cm of the soil was sampled after removal of the litter layer. The soil was dried at 40°C, sieved over 2 mm for homogeneity and stored at 4°C until further use.

Soil samples of 75 g dry soil were pre-incubated in glass jars for 7 days, at 16°C and 40% water holding capacity (WHC). Temperature and moisture conditions during incubation were set at 16°C and 80% WHC, respectively. The incubation period for the experiments was 28 h, as preliminary experiments had shown such a period to allow for sufficient \(\text{N}_2\text{O}\) production.

At the start of the incubation, all samples received 100 mg N kg\(^{-1}\) soil consisting of 50 mg NH\(_{4}\)-N kg\(^{-1}\) and 50 mg NO\(_{3}\)-N kg\(^{-1}\) soil. Four different treatments were established, each replicated five times. The different treatments (TR) involved the application of compounds enriched in \(^{18}\text{O}\) or \(^{15}\text{N}\), as follows:
\(^{18}\text{O}\) enriched \(\text{H}_2\text{O}\) (TR1), \(^{18}\text{O}\) enriched \(\text{NO}_3^-\) (TR2), \(^{15}\text{N}\) enriched \(\text{NO}_3^-\) (TR3), or \(^{15}\text{N}\) enriched \(\text{NH}_4^+\) (TR4). The respective compounds were enriched in \(^{18}\text{O}\) at 1.0 atom\% excess and at 40.0 atom\% excess for \(^{15}\text{N}\). The \(\text{NH}_4^+\) (\(^{15}\text{N}\) enriched and non-enriched) was applied as \(\text{NH}_4\text{Cl}\); \(\text{NO}_3^-\) as \(\text{Ca(NO}_3)_2\text{.4H}_2\text{O}\) (\(^{15}\text{N}\) enriched and non-enriched) and partially as \(\text{NaNO}_3\) in TR2 (\(^{18}\text{O}\) enriched \(\text{NO}_3^-\)). Demineralized water was used in all treatments to establish the correct moisture content. After treatment application, all jars were closed with septum-equipped lids for the duration of incubation.

At the end of incubation, gas samples were taken from the headspace and transferred to (vacuum) \(12\text{mL}\) exetainers, to be analyzed on \(\text{N}_2\text{O}\) content and its \(^{18}\text{O}\) signature. Subsequently, the soil dry weight of each replicate sample was determined to calculate its exact moisture content and therewith the exact \(^{18}\text{O}\) enrichment of the soil moisture during incubation.

Table 3.1: Description of the 12 soils studied, sampled across Europe. F, G and A denote forest, grassland and arable soils, respectively.

<table>
<thead>
<tr>
<th>Code</th>
<th>Soil texture</th>
<th>Latitude/longitude</th>
<th>Country</th>
<th>pH ((\text{H}_2\text{O}))</th>
<th>Vegetation and crops</th>
<th>Fertilizer(^b) (kgN ha(^{-1}) yr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>loam</td>
<td>48°30/11°11</td>
<td>GE</td>
<td>3.3(^c)</td>
<td>Norway Spruce</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td>sandy loam</td>
<td>61°51/24°17</td>
<td>FI</td>
<td>3.6</td>
<td>Scots Pine</td>
<td>0</td>
</tr>
<tr>
<td>F3</td>
<td>sandy loam</td>
<td>55°29/11°38</td>
<td>DE</td>
<td>4.2</td>
<td>Beech</td>
<td>0</td>
</tr>
<tr>
<td>F4</td>
<td>sandy loam</td>
<td>52°22/05°32</td>
<td>NL</td>
<td>3.8</td>
<td>Douglas fir, Oak</td>
<td>0</td>
</tr>
<tr>
<td>G1</td>
<td>sand</td>
<td>46°41/19°36</td>
<td>HU</td>
<td>7.8</td>
<td>Festuca spp.</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>clay</td>
<td>47°17/07°44</td>
<td>SW</td>
<td>6.0</td>
<td>Grass, Clover</td>
<td>150</td>
</tr>
<tr>
<td>G3</td>
<td>silt loam</td>
<td>55°52/03°12</td>
<td>UK</td>
<td>6.2</td>
<td>Lolium perenne</td>
<td>120</td>
</tr>
<tr>
<td>G4</td>
<td>silt loam</td>
<td>55°52/03°12</td>
<td>UK</td>
<td>5.9</td>
<td>Lolium perenne</td>
<td>290</td>
</tr>
<tr>
<td>A1</td>
<td>silty clay loam</td>
<td>51°06/10°55</td>
<td>GE</td>
<td>7.1</td>
<td>Sugarbeet, winter Wheat</td>
<td>100</td>
</tr>
<tr>
<td>A2</td>
<td>silt loam</td>
<td>48°51/01°58</td>
<td>FR</td>
<td>7.2</td>
<td>Mustard, Maize, Wheat, Barley</td>
<td>175</td>
</tr>
<tr>
<td>A3</td>
<td>sandy clay</td>
<td>40°31/14°57</td>
<td>IT</td>
<td>7.5</td>
<td>Maize, Alfalfa, Lolium perenne</td>
<td>500</td>
</tr>
<tr>
<td>A4</td>
<td>clay loam</td>
<td>45°12/09°04</td>
<td>IT</td>
<td>7.1</td>
<td>Maize, Rice</td>
<td>400, 100(^d)</td>
</tr>
</tbody>
</table>

\(^a\) USDA soil texture classification
\(^b\) approximate, mineral plus organic fertilizer-N
\(^c\) pH measured in CaCl\(_2\)
\(^d\) fertilizer for maize, rice respectively
Isotopic analyses

Gas samples were analyzed at the UC Davis Stable Isotope Facility. The N₂O concentration and its ¹⁵N and ¹⁸O signatures were determined using a Sercon Cryoprep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Isotope ratios were compared with N₂ and N₂O reference gases injected into the mass spectrometer within each sample analysis. Results were normalized using correction factors derived for standard samples containing 1000 μL m⁻³ N₂O balanced with N₂, which were distributed throughout the analytical batch. No international certified isotope standards are available for N₂O; therefore, we calibrated the δ¹⁵N of the N₂O reference gas by comparison with N₂ with known isotopic content (i.e. δ¹⁵N = -3.1‰ vs. atmospheric N₂) after reduction of N₂O to N₂ over copper at 600°C. We derived a δ¹⁸O value for the N₂O reference gas by comparison with CO₂ of known isotopic content (δ¹⁸O = 10.41‰ VSMOW) after conversion of both gases to CO over carbon at 1400°C. These measurements showed good precision for δ¹⁵N in N₂O (standard deviation = 0.06‰ (n=8)) and greater variability for ¹⁸O in N₂O (standard deviation = 0.96‰ (n=8)). We do not report ¹⁵N and ¹⁸O signatures when the N₂O concentrations were below 800 and 5000 μL m⁻³, respectively, as we considered these values to be the lower threshold values for reliable analysis. The above gas concentrations correspond to 0.4 and 2.5 nmol N₂O in the gas samples. At these amounts of N₂O, the typical standard deviation of isotope measurements is approximately 3‰.

Data calculations

The use of enriched compounds allowed us to consider only reaction stoichiometry and O exchange as determinants of the ¹⁸O signature of N₂O, as the effect of isotope fractionation would be negligible.

Presence of O exchange would be confirmed when the ¹⁸O incorporation from H₂O into N₂O (TR1) exceeded the calculated maximum incorporation that could be achieved through reaction stoichiometry. In the absence of O exchange, only N₂O derived from NH₄⁺ would contain ¹⁸O originating from H₂O. According to reaction stoichiometry, NO₃⁻ produced through nitrification of NH₄⁺ obtains two of its three O-atoms from H₂O (Figure 3.1). The same 2:3 ratio would hold for the
N$_2$O subsequently produced by denitrification of this nitrification-derived NO$_3^-$.
When produced through nitrifier denitrification, the N$_2$O (resulting from NO$_2^-$
reduction) obtains half of its O atoms from O$_2$ and half from H$_2$O during
oxidation of NH$_4^+$ to NO$_2^-$ (Figure 3.1). The maximum incorporation based on
reaction stoichiometry is therefore calculated by assuming that all NH$_4^+$
derived N$_2$O is produced through nitrification-coupled denitrification. As such, the
$^{18}$O enrichment of N$_2$O could reach maximally 2/3 of the $^{18}$O enrichment of the
applied H$_2$O:

$$\text{Maximum }^{18}O\text{ incorporation (\%) } = \frac{2}{3} \cdot {^{18}O(H_2O)} \cdot {^{18}O(NH_4)}$$  \hspace{1cm} \text{(eq 3.1)}

where the $^{18}O(H_2O)$ is the O-enrichment of the applied H$_2$O (atom% excess), and
$^{18}O(NH_4)$ the percentage of NH$_4^+$-derived N$_2$O:

$$N_2O^{(NH_4)} = 100 \cdot \frac{^{15}N\left(N_2O^{(TR4)}\right)}{^{15}N\left(N_2O^{(TR3)}\right) + ^{15}N\left(N_2O^{(TR4)}\right)}$$  \hspace{1cm} \text{(eq 3.2)}

with $^{15}N(N_2O^{(TR3)})$ and $^{15}N(N_2O^{(TR4)})$ denoting the $^{15}$N enrichment (atom% excess)
of the N$_2$O in treatment TR3 and TR4, respectively (Table 3.2).

Application of both $^{18}$O and $^{15}$N enriched NO$_3^-$ enabled the quantification of O
exchange during denitrification. If no $^{18}$O from NO$_3^-$ would be exchanged with
(non-enriched) H$_2$O-O during denitrification, the $^{18}$O:$^{15}$N ratio of NO$_3^-$ should be
retained in N$_2$O, and all intermediates. Note that a dilution of the (intermediate)
compounds would affect both enrichments equally, and therefore would not
change their ratio. The $^{18}$O:$^{15}$N enrichment ratio retention (ERR) in the N$_2$O
compared to NO$_3^-$ should therefore be 100% in the absence of O exchange:

$$\text{ERR (\%) } = 100 \cdot \frac{^{18}O\left(N_2O^{(TR2)}\right)}{^{18}O\left(NO_3^-(TR2)\right)}$$  \hspace{1cm} \text{(eq 3.3)}

where $^{18}O(N_2O^{(TR2)})$ denotes the $^{18}$O enrichment of the N$_2$O produced in treatment
TR2, and $^{18}O(NO_3^-(TR2))$ and $^{15}N(NO_3^-(TR3))$ the $^{18}$O and $^{15}$N enrichment of the NO$_3^-$
applied in treatment TR2 and TR3, respectively.

The loss of the $^{18}$O enrichment relative to the $^{15}$N from NO$_3^-$ into N$_2$O consequently quantifies the percentage of O that has been exchanged ($X_{ERR}$):

$$X_{ERR} = 100 - \text{ERR}$$  \hspace{1cm} \text{(eq 3.4)}
The $^{18}$O signature of biogenic N$_2$O is determined by O exchange with water

**Results and Discussion**

In all soils the measured incorporation of O from $^{18}$O enriched H$_2$O into N$_2$O exceeded the calculated maximum based on reaction stoichiometry, thereby confirming the presence and significance of O exchange during denitrification (Figure 3.2). Furthermore, the $^{18}$O:$^{15}$N enrichment ratio retention (ERR) from NO$_3^-$ into N$_2$O was incomplete for all soils (Figure 3.3), demonstrating O exchange during NO$_3^-$ reduction. The median O exchange was 85%, indicating that substantial O exchange during denitrification readily occurs in most, if not all, soils (Figure 3.3 and 3.4). The extent of exchange was relatively low for soils F3 and F4, where N$_2$O production was only marginally above background levels (Table 3.2, Figure 3.4). We conclude that in soils exhibiting significant N$_2$O production, O exchange between H$_2$O and intermediates of (de)nitrification will be a widespread feature and therefore largely determine the O isotopic composition of the N$_2$O.

Table 3.2: Average N$_2$O production and its relevant isotopic enrichment for each treatment ($^{18}$O or $^{15}$N). Production is averaged for samples across all treatments. The standard errors of the mean are given between brackets. F, G and A denote forest, grassland and arable soils, respectively.

<table>
<thead>
<tr>
<th>Code</th>
<th>N$_2$O production$^a$ (μgN$_2$O-N kg$^{-1}$ soil)</th>
<th>Isotopic enrichment</th>
<th>TR1 ($^{18}$O at%exc$^b$)</th>
<th>TR2 ($^{18}$O at%exc$^b$)</th>
<th>TR3 ($^{15}$N at%exc$^b$)</th>
<th>TR4 ($^{15}$N at%exc$^b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.7 (0.3)</td>
<td>0.676 (0.092)</td>
<td>0.073 (0.002)</td>
<td>13.38 (2.02)</td>
<td>0.27 (0.05)</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>21.6 (2.5)</td>
<td>0.853 (0.015)</td>
<td>0.152 (0.003)</td>
<td>54.44 (0.97)</td>
<td>0.03 (0.01)</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>0.5 (0.0)</td>
<td>0.164 (0.042)</td>
<td>0.135 (0.049)</td>
<td>6.10 (0.69)</td>
<td>0.25 (0.08)</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>0.3 (0.0)</td>
<td>0.284 (0.052)</td>
<td>0.106 (0.022)</td>
<td>5.46 (1.23)</td>
<td>0.22 (0.08)</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>26.2 (9.8)</td>
<td>0.764 (0.014)</td>
<td>0.052 (0.008)</td>
<td>3.04 (0.28)</td>
<td>26.92 (1.35)</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>1031.0 (74.2)</td>
<td>0.896 (0.003)</td>
<td>0.064 (0.002)</td>
<td>26.50 (1.89)</td>
<td>0.70 (0.05)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>46.2 (13.7)</td>
<td>0.634 (0.137)</td>
<td>0.056 (0.011)</td>
<td>10.47 (1.22)</td>
<td>7.99 (2.55)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>924.3 (122.9)</td>
<td>0.868 (0.009)</td>
<td>0.090 (0.004)</td>
<td>20.27 (0.24)</td>
<td>0.52 (0.21)</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>239.1 (20.6)</td>
<td>0.914 (0.011)</td>
<td>0.027 (0.002)</td>
<td>24.41 (0.18)</td>
<td>3.20 (0.15)</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>219.5 (27.3)</td>
<td>0.995 (0.003)</td>
<td>0.016 (0.001)</td>
<td>19.54 (0.20)</td>
<td>6.06 (0.07)</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>95.4 (10.8)</td>
<td>0.958 (0.003)</td>
<td>0.017 (0.001)</td>
<td>14.31 (0.07)</td>
<td>6.09 (0.17)</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>146.3 (14.3)</td>
<td>0.912 (0.004)</td>
<td>0.060 (0.002)</td>
<td>18.82 (0.14)</td>
<td>2.95 (0.04)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ average for all treatments
$^b$ at%exc = atom % excess
To be complete, when interpreting the ERR we need to reflect on the potential of N$_2$O production through co-denitrification as well. N$_2$O derived through this process may have obtained (maximally) one of its N from another source, while all O may still originate from the (18O enriched) NO$_3^-$. The measured relative loss in the 18O:15N enrichment ratio could thus be partially due to a dilution of the 15N enrichment, which would imply a lower extent of O exchange than quantified with the current ERR assumptions. However, though the capacity for co-denitrification has been identified for fungi and bacteria (Garber et al., 1982; Kim et al., 1984; Tanimoto et al., 1992b; Morozkina et al., 2007), its significance in total N$_2$O production in soil has not been identified yet. As we currently quantify a median exchange of 85%, even if part of the loss in ERR should be ascribed to co-denitrification, the extent of O exchange remains highly significant.

Despite the diversity of the soils and land use types, O exchange occurred in all these soils. This suggests that O exchange is a universal feature of biogenic N$_2$O production. As O isotopic analyses of N$_2$O are employed in source determination studies outside soil ecosystems as well, we argue that implications of O exchange need to be considered across aqueous and atmospheric ecosystems, as the same microbial processes are responsible for N$_2$O production in those systems as well.

Figure 3.2: Oxygen incorporation from H$_2$O into N$_2$O for all soils (%). Grey bars present the actual O incorporation measured, white bars present the theoretical maximum in absence of O exchange. The actual amount of O incorporated from H$_2$O into N$_2$O exceeds the theoretical maximum for all soils. These maxima are based on reaction stoichiometry and the relative contribution of NH$_4^+$ and NO$_3^-$ to total N$_2$O production, deduced from the 15N enrichment data (Table 3.2).
The $^{18}$O signature of biogenic N$_2$O is determined by O exchange with water.

In aqueous systems such as lakes, marine environments and wastewater treatment plants, characterization of O isotopic signatures of N$_2$O has been carried out to study its sources and sinks (Wahlen et al., 1985; Yoshinari et al., 1985; Kim et al., 1990; Yoshinari et al., 1997; Naqvi et al., 1998). The O isotopic composition of N$_2$O is thereby assumed to depend on the $^{18}$O signatures of O$_2$ and H$_2$O during N$_2$O formation through nitrification and denitrification (in ratios reflecting reaction stoichiometry) and on fractionation upon its reduction to N$_2$ (Kim et al., 1990; Yoshinari et al., 1997). However, if H$_2$O is effectively the major O source of N$_2$O through the presence of O exchange as in our study, the interpretation of the O isotopic signatures in such studies needs to be reconsidered.

In the atmosphere, the O isotopic signature of N$_2$O will scarcely be affected by O exchange in situ. Despite the presence of active microorganisms in the atmosphere (Dimmick et al., 1979; Amato et al., 2007), N$_2$O is thought to be almost exclusively produced at the earth surface. Microbial nitrification and

Figure 3.3: The $^{18}$O and $^{15}$N isotopic enrichment of N$_2$O produced from denitrification of applied NO$_3^-$ (TR2 and TR3 for $^{18}$O and $^{15}$N respectively). When no O exchange occurs, the ratio of enrichments of the applied NO$_3^-$, represented by the solid line, should be retained in the produced N$_2$O (ERR = 100%). Data points for all soils are positioned below this line, denoting a loss in $^{18}$O enrichment relative to $^{15}$N in N$_2$O compared to the NO$_3^-$ and demonstrating the presence of O exchange. The dashed lines indicate the minimum, maximum and median exchange measured in these soils.
Denitrification in soils and water are considered to be the main sources of atmospheric N₂O, with some minor contribution from biomass burning, industry, combustion in vehicles and power plants (Stein et al., 2003; Kaiser et al., 2005; Bernard et al., 2006; IPCC, 2007; Sorai et al., 2007). In the atmosphere, direct O exchange between H₂O and N₂O is unlikely (Wahlen et al., 1985; Cliff et al., 1997) and is experimentally proven to be negligible for the reaction N₂O + O(1D) in particular (Kaiser et al., 2005). However, the N₂O emitted from terrestrial and possibly aqueous ecosystems to the atmosphere will have been subject to O exchange during its biochemical production. This corroborates with the observation that the identified ¹⁷O anomaly of atmospheric N₂O can be adequately explained by a balanced budget combining biological N₂O emissions and several chemical production sources (Kaiser et al., 2005), under the assumption that H₂O is the only source of O in the microbiologically produced N₂O, providing the N₂O with the same ¹⁶O/¹⁷O/¹⁸O-isotope signature as H₂O (Meijer et al., 1998; Kaiser et al., 2004). It remains striking that while these atmospheric studies assume the O in N₂O from the Earth surface is exclusively derived from H₂O, terrestrial and aquatic studies generally assume that the N₂O-O is derived from both O₂ and H₂O in ratios reflecting reaction stoichiometry (Kim et al., 1990; Yoshinari et al., 1997; Pérez, 2005). Our results, though soil-derived, corroborate with the former assumption rather than the latter.

Figure 3.4: The O exchange during denitrification versus N₂O production for all soils. The amount of exchange is calculated using the enrichment ratio retention (ERR) method. The N₂O production is averaged over all replicates of the treatments used for the O exchange calculation (TR2 and TR3).
The $^{18}$O signature of biogenic N$_2$O is determined by O exchange with water.

In addition, we suggest that O exchange might affect not only the N$_2$O, but also the intermediate compounds of its production. Future studies should therefore reflect on potential implications of O exchange for NO$_3^-$ source determination based on O isotopic analyses as well.

In summary, our results show that H$_2$O constitutes the main source of O in N$_2$O and possibly other nitrogen oxides as well. Nevertheless, such compounds exhibit wide ranges in its O isotopic signature in different pools and sources. For N$_2$O emissions from soils alone, the reported $\delta^{18}$O values range from 19.6 to 57.8‰ (Pérez, 2005). However, these large ranges can, partly, be explained by the lack of an international standard. Moreover, the O isotopic signature of H$_2$O itself (in precipitation, ground water, river water or even tap water) also varies widely across temporal and spatial scales (Dutton et al., 2005; Reddy et al., 2006; Bowen et al., 2007).

**Conclusion**

We conclude that up to 100% of the O in N$_2$O can be derived from H$_2$O through O exchange. Our results prove that general assumptions on the origin of the O and the consequent O isotopic signature of N$_2$O (Kim et al., 1990; Pérez, 2005) do not hold. O$_2$ as a source of N$_2$O-O may often be negligible; in N$_2$O production by denitrifiers, the O isotopic signature of N$_2$O does not necessarily reflect that of the substrate NO$_3^-$ at all. The evident significance of O exchange during N$_2$O production poses a global challenge for the use and interpretation of O isotopic analyses in biogeochemical studies of the N cycle in the biosphere.

**Acknowledgements**

We like to thank T. Röckmann, W. De Vries and P.C. De Ruiter for critical comments on previous versions of the manuscript, and S. Zechmeister-Boltenstern for coordinating the soil sampling campaign. This research was financed by the NitroEurope IP (GOCE-017841), funded by the European Commission under the 6th framework program. J.W. Van Groenigen is supported by a personal VIDI grant from the Netherlands Organization of Scientific Research/Earth and Life Sciences (NWO-ALW).
Abstract  Interpretation of the oxygen isotopic signature of soil-derived N\textsubscript{2}O may be flawed when it is based on reaction stoichiometry and fractionation alone. In fact, oxygen (O) exchange between H\textsubscript{2}O and intermediates of N\textsubscript{2}O production pathways may largely determine this O isotopic signature. Although in our previous work we conclusively proved the occurrence of O exchange during N\textsubscript{2}O production by denitrification of NO\textsubscript{3} -, its occurrence in N\textsubscript{2}O production pathways by nitrifiers remains unclear. The aim of this study was to examine the likeliness of O exchange during various stages of N\textsubscript{2}O production in soil via nitrification, nitrifier denitrification and denitrification. We evaluated a set of scenarios on the presence of such exchange using data from a series of 18O and 15N tracing experiments. The measured actual O incorporation from H\textsubscript{2}O into N\textsubscript{2}O (AOI) was compared with the theoretical maximum O incorporation (MOI) from various scenarios that differed in their assumptions on the presence of O exchange. We found that scenarios where O exchange was assumed to occur exclusively during denitrification could not explain the observed AOI, as it exceeded the MOI for 9 out of 10 soils. This demonstrates that additional O exchange must have occurred in N\textsubscript{2}O production through nitrifier pathways. It remains to be determined in which steps of these pathways O exchange can take place. We conclude that O exchange is likely to be mediated by ammonia oxidizers during NO\textsubscript{2} - reduction (nitrifier denitrification), and that it could possibly occur during NO\textsubscript{2} oxidation to NO\textsubscript{3} by nitrite oxidizers as well.

D.M. Kool, C. Müller, N. Wrage, O. Oenema, J.W. Van Groenigen. 2009. Oxygen exchange between nitrogen oxides and H\textsubscript{2}O can occur during nitrifier pathways. Soil Biology and Biochemistry 41: 1632-1641
Chapter 4

Introduction

Nitrous oxide (N₂O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). The rising of its atmospheric concentrations, primarily caused by anthropogenic activities, has led to the demand for measures that adequately mitigate the emissions of N₂O into the atmosphere. Soils comprise the major source of atmospheric N₂O (IPCC, 2007), and accurate understanding of its biochemical production pathways in soil is therefore key to the development of adequate mitigation strategies. Nitrification, nitrifier denitrification, and denitrification have been identified as the major microbial N₂O producing pathways in terrestrial and aquatic ecosystems (Firestone et al., 1989; Granli et al., 1994; Wrage et al., 2001). Given the reaction stoichiometry of these pathways, the relative contribution of O₂ and H₂O as sources of the oxygen (O) in N₂O differs between these production pathways (Figure 4.1). The O isotopic signatures of N₂O are accordingly assumed to be distinct for these different pathways, providing O isotopic analysis of N₂O as a promising and increasingly used tool in studying its sources and production processes (Naqvi et al., 1998; Pérez, 2005; Wrage et al., 2005). Unfortunately, the interpretation of the O isotopic signature based on reaction stoichiometry (and fractionation effects) alone, may be significantly flawed because of O exchange between H₂O and intermediates of the production pathways (Kool et al., 2007; Kool et al., 2009a). In the few studies where it has been considered, O exchange was typically assumed to be negligible (Wahlen et al., 1985; Toyoda et al., 2005; Wrage et al., 2005; Menyailo et al., 2006a). However, based on a literature review we recommended that the O isotopic signature of N₂O should be interpreted with extreme caution because of the probability of O exchange (Kool et al., 2007). We recently showed experimentally, using a combination of O and N isotope tracing, that O exchange during N₂O production was highly significant in a wide range of soils (Kool et al., 2009a). Such exchange between H₂O and intermediates of N₂O production took place in all soils studied, with a median of 85% oxygen exchanged. We therefore concluded that the occurrence of O exchange needs to be taken into account to correctly interpret the O isotopic signature of N₂O (Kool et al., 2009a).

However, it is not clear during what processes and at which stages of N₂O
Oxygen exchange between nitrogen oxides and H$_2$O during nitrifier pathways

production the O exchange occurs. The study by Kool et al. (2009a) confirmed its presence during denitrification of NO$_3^-$ to N$_2$O (Figure 4.1, i). This will affect the O isotopic signature of N$_2$O produced through denitrification of NO$_3^-$ generated through nitrification (nitrification-coupled denitrification, NCD), as well as from applied fertilizer NO$_3^-$ (fertilizer denitrification, FD) (Figure 4.1). The widespread occurrence and high rates of O exchange during denitrification raises the question whether such O exchange might also be present during nitrifier-mediated N$_2$O formation. Oxygen exchange in nitrifier pathways may be anticipated because several reaction steps featuring in these pathways occur in the NO$_3^-$ denitrification pathway as well. Two distinct processes may be eligible to facilitate O exchange: the reduction of NO$_2^-$ to N$_2$O by ammonium oxidizers (Figure 4.1, ii); and NO$_2^-$ oxidation to NO$_3^-$ by nitrite oxidizers (Figure 4.1, iii). The first process would affect the O in N$_2$O produced through nitrifier denitrification (ND). Through the latter process, N$_2$O produced by NCD would be affected, and potentially by ND as well. For both processes, similarities are found between the enzymes that catalyze these reaction steps when carried out by nitrifiers and
denitrifiers. In ammonia oxidizers carrying out nitrifier denitrification (Figure 4.1, ii), the enzyme that reduces \( \text{NO}_2^- \) to NO is a copper-containing nitrite reductase (NiR) similar to the copper-NiR found in denitrifiers (Casciotti et al., 2001; Chain et al., 2003; Cantera et al., 2007; Garbeva et al., 2007). Also the genes encoding for NO reductase (NOR) involved in NO reduction by these nitrifiers appear analogous to those in denitrifiers (Casciotti et al., 2005; Garbeva et al., 2007). The oxidation of \( \text{NO}_2^- \) to \( \text{NO}_3^- \) by nitrite oxidizers (Figure 4.1, iii) is catalyzed by the enzyme nitrite oxidoreductase (Aleem, 1968; Sundermeyer-Klinger et al., 1984; Wood, 1986). This enzyme is found to be a molybdenum iron-sulfur complex, which is also the case for nitrate reductases that catalyze the reverse reaction in denitrifiers (Satoh, 1981; Sundermeyer-Klinger et al., 1984). As the O exchange during these transformations is likely to be a biochemical process, the similarity between the enzymes employed by nitrifiers and denitrifiers suggests that O exchange could occur in these nitrifier pathways as well.

Summarizing, in order to properly interpret the O isotopic signature of \( \text{N}_2\text{O} \), we should explore the potential of O exchange during all \( \text{N}_2\text{O} \) producing pathways. In this paper we evaluate the likeliness of O exchange between \( \text{H}_2\text{O} \) and intermediates of the major \( \text{N}_2\text{O} \) production pathways. Our evaluation is based on the analysis of the incorporation of O from \(^{18}\text{O} \) enriched \( \text{H}_2\text{O} \) and \(^{18}\text{O} \) enriched \( \text{NO}_3^- \) into produced \( \text{N}_2\text{O} \) that was measured during soil incubation experiments. These results are compared with theoretical maxima of O incorporation for a series of scenarios that consider the occurrence of O exchange during the various \( \text{N}_2\text{O} \) producing pathways. The use of enriched compounds in the incubation experiments allowed us to disregard isotope fractionation and to focus on reaction stoichiometry and O exchange.

**Methods**

*Soil incubation*

Soil samples from 12 sites across Europe were collected for the soil incubation experiment (Kool et al., 2009a). The soils originated from forest, grassland, and arable fields, the main land uses across Europe (Table 4.1). The experimental units consisted of soil samples (75g) which were pre-incubated at 16°C and 40% water holding capacity (WHC) a week prior to the incubation. At the start of the
incubation, experimental units received different combinations of $^{18}$O and $^{15}$N labeled compounds. All units received equal total amounts of mineral N (50 mg NH$_4^+$-N kg$^{-1}$ and 50 mg NO$_3^-$-N kg$^{-1}$ soil), were incubated at 80% WHC by adding appropriate amounts of H$_2$O, and the temperature was kept at 16°C. The following four treatments with isotopically enriched compounds were implemented: $^{18}$O enriched H$_2$O at 1.0 atom% excess (TR1), $^{18}$O enriched NO$_3^-$ at 1.0 atom% excess (TR2), $^{15}$N enriched NO$_3^-$ at 40.0 atom% excess (TR3), and $^{15}$N enriched NH$_4^+$ at 40.0 atom% excess (TR4). The experiment was set up as a completely randomized design, with five replicates for each of the four treatments. The jars were closed, by lids equipped with rubber septa, for an incubation period of 28h. At the end of the incubation, gas and soil samples were taken. Gas samples were extracted from the headspace and transferred to 12mL exetainers that were flushed with helium and evacuated before use. The N$_2$O

Table 4.1: Description of the 12 soils incubated, sampled across Europe. F, G and A denote forest, grassland and arable soils, respectively. Soils F3 and F4 were excluded from the scenario evaluation as their total N$_2$O production during incubation was only marginally above background levels.

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Soil texture</th>
<th>Latitude/longitude</th>
<th>Country</th>
<th>pH (H$_2$O)</th>
<th>Vegetation and crops</th>
<th>Fertilizer$^a$ (kgN ha$^{-1}$ yr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 loam</td>
<td>48°30/11°11</td>
<td>GE</td>
<td>3.3$^b$</td>
<td>Norway Spruce</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2 sandy loam</td>
<td>61°51/24°17</td>
<td>FI</td>
<td>3.6</td>
<td>Scots Pine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3 sandy loam</td>
<td>55°29/11°38</td>
<td>DE</td>
<td>4.2</td>
<td>Beech</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4 sandy loam</td>
<td>52°22/05°32</td>
<td>NL</td>
<td>3.8</td>
<td>Douglas fir, Oak</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 sand</td>
<td>46°41/19°36</td>
<td>HU</td>
<td>7.8</td>
<td>Festuca spp.</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2 clay</td>
<td>47°17/07°44</td>
<td>SW</td>
<td>6.0</td>
<td>Grass, Clover</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3 silt loam</td>
<td>55°52/-03°12</td>
<td>UK</td>
<td>6.2</td>
<td>Lolium perrenne</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4 silt loam</td>
<td>55°52/-03°12</td>
<td>UK</td>
<td>5.9</td>
<td>Lolium perrenne</td>
<td>290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 silty clay loam</td>
<td>51°06/10°55</td>
<td>GE</td>
<td>7.1</td>
<td>Sugarbeet, winter Wheat</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2 silt loam</td>
<td>48°51/01°58</td>
<td>FR</td>
<td>7.2</td>
<td>Mustard, Maize, Wheat, Barley</td>
<td>175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 sandy clay</td>
<td>40°31/14°57</td>
<td>IT</td>
<td>7.5</td>
<td>Maize, Alfalfa, Lolium perrenne</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4 clay loam</td>
<td>45°12/09°04</td>
<td>IT</td>
<td>7.1</td>
<td>Maize, Rice</td>
<td>400, 100$^d$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ USDA soil texture classification
$^b$ approximate, mineral plus organic fertilizer-N
$^c$ pH measured in CaCl$_2$
$^d$ fertilizer for maize, rice respectively
concentration and its isotopic signature were measured at the UC Davis Stable Isotope Facility, using a Sercon Cryo prep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Further details on the gas sampling and analyses for N₂O production and isotopic signature were described previously (Kool et al., 2009a).

Soil samples were taken after gas sampling. Sub-samples of the soil were taken to determine the soil moisture content and the exact ¹⁸O enrichment of the soil water. Only minor changes in the moisture content over the incubation period were observed. Other sub-samples of approximately 20 g moist soil were taken for analyses of mineral N (NH₄⁺-N and NO₃⁻-N) and its ¹⁵N isotopic signature. Soil mineral N content was determined by extraction with 1M KCl (50 mL 20g⁻¹ soil) followed by segmented flow analyses (SFA) (Skalar Analytical, Breda, The Netherlands) (Kool et al., 2006).

The ¹⁵N enrichments of the mineral N were derived using a microdiffusion method based on Van Groenigen et al. (2005b). In short, for the NH₄⁺ isolation a microfilter spiked with KHSO₄ (2M) and packed in Teflon was added to the sample, together with ashed MgO to raise the pH to approximately 10, and the sample containers were closed for (at least) 6 days. The filter was removed before the addition of Devarda’s alloy, and a new filter for the NO₃⁻ isolation was added. The samples were left at room temperature (20°C) for both microdiffusion steps. The isotopic analyses were carried out at UC Davis SIF on an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (EA-IRMS) (Sercon 20/20, Sercon Ltd., Cheshire, UK). Two laboratory standards were analyzed with every 12 samples. The laboratory samples were calibrated against NIST standard reference materials.

Data calculations

The measured N₂O production, its ¹⁸O and ¹⁵N signatures and the ¹⁵N signatures of NH₄⁺ and NO₃⁻ provided the input for our calculations. The isotopic signatures of the soil mineral N used are the average enrichments over the incubation period, calculated by assuming linear changes in enrichment of the pools. Of the 12 soils used in the incubation, two (F3 and F4) were excluded from further analysis as their total N₂O production was only marginally above background levels (Kool et al., 2009a).
The $^{18}$O incorporation from H$_2$O into N$_2$O, derived from TR1, was calculated previously in Kool et al. (2009a). It is here referred to as the ‘actual O incorporation’ (AOI, in %), and was calculated as follows:

$$AOI = 100 \cdot \frac{^{18}O(N_{2}O_{(TR1)})}{^{18}O(H_{2}O_{(TR1)})}$$  \hspace{1cm} \text{(eq 4.1)}

where $^{18}O(N_{2}O_{(TR1)})$ and $^{18}O(H_{2}O_{(TR1)})$ denote the O isotopic enrichment (atom% excess) of the produced N$_2$O and the soil H$_2$O, respectively, in TR1.

The extent of O exchange ($X_{ERR}$) for all soils was calculated using the $^{18}$O:$^{15}$N enrichment ratio retention (ERR) method (Kool et al., 2009a). This ERR method quantifies the exchange by comparing the ratio of $^{18}$O and $^{15}$N enrichment in the produced N$_2$O with the ratio at which it was applied in NO$_3$-. In other words, this ERR is the percentage (%) of the ratio of $^{18}$O:$^{15}$N enrichment in the NO$_3^-$ that is retained in the N$_2$O:

$$ERR = 100 \cdot \frac{^{18}O(N_{2}O_{(TR2)})}{^{15}N(N_{2}O_{(TR3)})} / \frac{^{18}O(NO_{3}^-_{(TR2)})}{^{15}N(NO_{3}^-_{(TR3)})}$$  \hspace{1cm} \text{or}:

$$ERR = 100 \cdot \frac{^{18}O(N_{2}O_{(TR2)})}{^{15}N(N_{2}O_{(TR3)})} \cdot \frac{^{15}N(NO_{3}^-_{(TR3)})}{^{18}O(NO_{3}^-_{(TR2)})}$$  \hspace{1cm} \text{(eq 4.2)}

where $^{18}O(N_{2}O_{(TR2)})$ and $^{15}N(N_{2}O_{(TR3)})$ denote the O and N enrichment of the produced N$_2$O, and $^{18}O(NO_{3}^-_{(TR2)})$ and $^{15}N(NO_{3}^-_{(TR3)})$ the O and N enrichment of the applied NO$_3^-$ (atom% excess), in TR2 and TR3, respectively. This ratio of the $^{18}$O to the $^{15}$N enrichments should be conserved in the absence of O exchange, as it is not altered (through dilution) by N$_2$O production through other sources (i.e. an ERR of 100%). It will however decrease when O exchange occurs, as that would only alter the $^{18}$O enrichment and not the $^{15}$N. The loss in $^{18}$O enrichment relative to $^{15}$N from NO$_3^-$ into N$_2$O quantifies the percentage (%) of O that has been exchanged:

$$X_{ERR} = 100 - ERR$$  \hspace{1cm} \text{(eq 4.3)}
Chapter 4

The relative proportions of total N\textsubscript{2}O derived from NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−}, \(N_2O_{(NH4)}\) and \(N_2O_{(NO3)}\) respectively, follow from the \(^{15}\text{N}\) enrichment of the \(N_2O\) established in the treatments TR3 and TR4. They are calculated as percentage (%) of total \(N_2O\) production:

\[
N_2O_{(NH 4)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR 4)}\right)}{^{15}N\left(N_2O_{(TR 3)}\right) + ^{15}N\left(N_2O_{(TR 4)}\right)} \quad \text{(eq 4.4)}
\]

\[
N_2O_{(NO 3)} = 100 \cdot \frac{^{15}N\left(N_2O_{(TR 3)}\right)}{^{15}N\left(N_2O_{(TR 3)}\right) + ^{15}N\left(N_2O_{(TR 4)}\right)} \quad \text{(eq 4.5)}
\]

where \(^{15}N(N_2O_{(TR4)})\) refers to the \(^{15}\text{N}\) enrichment (atom% excess) of the \(N_2O\) in TR4.

**Oxygen exchange evaluation**

We explore the likeliness of O exchange during the various \(N_2O\) production pathways by evaluating the incorporation of O from H\textsubscript{2}O into \(N_2O\) (OI(N\textsubscript{2}O)). The actual OI (AOI) was measured in the incubation study. Next, the theoretical maximum OI (MOI) is calculated based on the maximum O incorporation that can be attained through reaction stoichiometry, plus the potential O exchange dependent on various scenarios. If the AOI exceeds MOI this implies that the measured \(^{18}\text{O}\) enrichment of the \(N_2O\) cannot be fully explained with the assumptions on O exchange under that scenario. Higher MOI that would better fit the observed AOI could be obtained when O exchange is assumed to be more abundant. Accordingly, a series of six scenarios A to F is constructed and evaluated, where O exchange is assumed to take place during one or more of the processes in \(N_2O\) production pathways (Figure 4.1).

**Oxygen exchange scenarios**

In the scenarios we considered the occurrence of O exchange during one or more of the following reaction steps (Figure 4.1):

i. NO\textsubscript{3}\textsuperscript{−} reduction by denitrifiers (denitrification);

ii. NO\textsubscript{2} reduction to \(N_2O\) by ammonia oxidizers (nitrifier denitrification);

iii. NO\textsubscript{2} oxidation to NO\textsubscript{3}\textsuperscript{−} by nitrite oxidizers (second part of nitrification).
For the process of NO$_3^-$ oxidation (iii), in the scenarios we made the distinction between O exchange that will effect the O isotopic composition of the product NO$_3^-$ only (iii-a), or for the NO$_2^-$ (substrate) as well (iii-b). The occurrence and extent of O exchange has already been established for (i), NO$_3^-$ reduction by denitrifiers (Kool et al., 2009a), and is thus included in all scenarios. Wherever additional O exchange is considered to be present, we assume that it takes place to the same extent as it was quantified for NO$_3^-$ reduction to N$_2$O ($X_{ERA}$). We calculate the theoretical maximum O incorporation (MOI) for six scenarios A through F, under which O exchange is assumed to occur as follows (Table 4.2):

A. only during the denitrification of NO$_3^-$ by denitrifiers (i);
B. during (i), and during nitrifier denitrification of NO$_2^-$ (ii)
C. during (i), and during oxidation of NO$_2^-$ to NO$_3^-$ by nitrifiers (iii), affecting only the NO$_3^-$ (iii-a);
D. during (i), and during oxidation of NO$_2^-$ to NO$_3^-$ by nitrifiers (iii), affecting both the NO$_2^-$ and NO$_3^-$ (iii-b);
E. during (i), during nitrifier denitrification of NO$_2^-$ (ii), and during oxidation of NO$_2^-$ to NO$_3^-$ by nitrifiers (iii), affecting only the NO$_3^-$ (iii-a);
F. during (i), during nitrifier denitrification of NO$_2^-$ (ii), and during oxidation of NO$_2^-$ to NO$_3^-$ by nitrifiers (iii), affecting both the NO$_2^-$ and NO$_3^-$ (iii-b).

Note that when all N$_2$O would be produced by denitrifiers (FD plus NCD), the O incorporation from H$_2$O into N$_2$O under scenario B would not differ from A, and that of D, E and F would not differ from that of C.

Table 4.2: Overview of the N-transformation processes during which O exchange is considered to occur under the different scenarios A to F. Figure 4.1 depicts the indicated processes, i.e. denitrification of NO$_3^-$ (i), nitrifier denitrification (ii), and NO$_2^-$ oxidation (iii). For iii, O exchange is assumed to affect only the product NO$_3^-$ under iii-a, and both NO$_3^-$ and the NO$_2^-$ under iii-b. A ‘V’ indicates that O exchange during the respective processes is included in the particular scenario.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>i</th>
<th>ii</th>
<th>iiia</th>
<th>iiib</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>V</td>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>V</td>
<td></td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>V</td>
<td>V</td>
<td></td>
<td>V</td>
</tr>
<tr>
<td>F</td>
<td>V</td>
<td>V</td>
<td></td>
<td>V</td>
</tr>
</tbody>
</table>

*'Figure 4.1 depicts the processes indicated as i, ii and iii
Oxygen incorporation calculation

The total O incorporation from H$_2$O into N$_2$O (OI(N$_2$O)) is determined by the O incorporation through each pathway (OI(N$_2$Op)), and by the relative contribution of the different pathways to total production (N$_2$Op). The extent of H$_2$O-O incorporation into N$_2$O for each pathway (OI(N$_2$Op)) times their relative contribution to total N$_2$O production (N$_2$Op) sum up to the total O incorporation from the applied enriched H$_2$O into N$_2$O:

\[
OI(N_2O) = \sum N_2O_p \cdot OI(N_2Op)
\]

\[
= N_2O_{FD} \cdot OI(N_2O_{FD}) + N_2O_{NCD} \cdot OI(N_2O_{NCD})
+ N_2O_{ND} \cdot OI(N_2O_{ND}) + N_2O_{NN} \cdot OI(N_2O_{NN})
\]

(eq 4.6)

Each OI(N$_2$Op) is a sum of the incorporation of H$_2$O-O through reaction stoichiometry plus additional incorporation through O exchange. Both differ for the various production pathways, the latter being constrained by the O exchange scenarios. The allocated N$_2$Op are constrained by the results of the soil incubation experiment. The different pathways facilitate different amounts of H$_2$O-O incorporation through reaction stoichiometry. To calculate the theoretical maximum OI(N$_2$O) (the MOI), the N$_2$Op of those pathways providing the highest OI(N$_2$Op) through reaction stoichiometry is maximized.

In the appendices, we describe in detail how the partial OI(N$_2$Op) and the relative contributions of the different pathways (N$_2$Op) are calculated for the respective scenarios (appendices A4-1 and A4-2, respectively). To evaluate the impact of maximizing the stoichiometric O incorporation, we explored two additional sub-evaluations S1 and S2 (appendix A4-2.3) where the contribution of the pathways providing highest OI(N$_2$Op) is not maximized. In the calculations, all OI(N$_2$Op) and the $X_{ERR}$ are inserted as fractions (range 0 to 1); the N$_2$Op, AOI and MOI are in percentages.

Sensitivity Analyses

In our evaluation it is assumed that O exchange takes place to the same extent as quantified for the denitrification pathway. We believe that this provides the most reasonable estimate of the extent of exchange. However, to further evaluate the
implications of this assumption, we carried out a sensitivity analysis of the parameter $X_{ERR}$ varying it by ± 10% (maximum value 100%) to evaluate resulting changes in the MOI.

**Results**

Table 4.3 lists the actual O incorporation from H$_2$O into N$_2$O (AOI) derived from treatment 1 (TR1), the extent of O exchange during denitrification ($X_{ERR}$) derived from TR2 and TR3, and the proportions of N$_2$O derived from NH$_4^+$ and NO$_3^-$ (derived from TR3 and TR4). All those parameters were calculated directly from data provided in Table 4.4, which presents the N$_2$O production and the relevant $^{18}$O and $^{15}$N signatures of N$_2$O and soil mineral N.

Averaged over all soils considered, 89.6% of O in the N$_2$O originated from H$_2$O. These levels of AOI confirm the presence of O exchange for all soils (Kool et al., 2009a). In general the data showed low levels of variation between replicates. However, two soils (F1 and G3) had relatively large SE values which could

<table>
<thead>
<tr>
<th>Soil</th>
<th>AOI</th>
<th>$X_{ERR}$</th>
<th>$N_{2}O_{(NO3)}$</th>
<th>$N_{2}O_{(NH4)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>79.0 (11.1)</td>
<td>78.3</td>
<td>98.02</td>
<td>1.98</td>
</tr>
<tr>
<td>F2</td>
<td>90.7 (1.8)</td>
<td>88.8</td>
<td>99.95</td>
<td>0.05</td>
</tr>
<tr>
<td>G1</td>
<td>74.6 (1.2)</td>
<td>32.2</td>
<td>10.16</td>
<td>89.84</td>
</tr>
<tr>
<td>G2</td>
<td>98.2 (0.4)</td>
<td>90.4</td>
<td>97.44</td>
<td>2.56</td>
</tr>
<tr>
<td>G3</td>
<td>65.4 (14.0)</td>
<td>78.7</td>
<td>56.71</td>
<td>43.29</td>
</tr>
<tr>
<td>G4</td>
<td>89.0 (1.0)</td>
<td>82.2</td>
<td>97.50</td>
<td>2.50</td>
</tr>
<tr>
<td>A1</td>
<td>95.9 (1.1)</td>
<td>95.6</td>
<td>88.43</td>
<td>11.57</td>
</tr>
<tr>
<td>A2</td>
<td>102.5 (0.3)</td>
<td>96.8</td>
<td>76.33</td>
<td>23.67</td>
</tr>
<tr>
<td>A3</td>
<td>104.9 (0.4)</td>
<td>95.3</td>
<td>70.14</td>
<td>29.86</td>
</tr>
<tr>
<td>A4</td>
<td>95.4 (0.4)</td>
<td>87.3</td>
<td>86.46</td>
<td>13.54</td>
</tr>
</tbody>
</table>

$^a$ mineral N
Table 4.4: Total N$_2$O production (µg N kg$^{-1}$ soil) and relevant O and N isotopic signatures (atom% excess) of N$_2$O and soil mineral N (NH$_4^+$, NO$_3^-$) of all soils considered in the assessment. Standard errors are given between brackets (n=20 for N$_2$O production, n=5 for all other data). In TR1, TR2, TR3 and TR4, soils received $^{18}$O enriched H$_2$O, $^{18}$O enriched NO$_3^-$, $^{15}$N enriched NO$_3^-$, and $^{15}$N enriched NH$_4^+$, respectively.

<table>
<thead>
<tr>
<th>Soil</th>
<th>N$_2$O production (µg N kg$^{-1}$ soil)</th>
<th>N$_2$O isotopic enrichment (atom% excess)</th>
<th>Soil mineral N isotopic enrichment (atom% excess)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TR1-N$_2$O ($^{18}$O)</td>
<td>TR2-N$_2$O ($^{18}$O)</td>
<td>TR3-NO$_3^-$ ($^{15}$N)</td>
</tr>
<tr>
<td>F1</td>
<td>1.7 (0.3)</td>
<td>0.68 (0.09)</td>
<td>0.07 (0.00)</td>
</tr>
<tr>
<td>F2</td>
<td>21.6 (2.5)</td>
<td>0.85 (0.01)</td>
<td>0.15 (0.00)</td>
</tr>
<tr>
<td>G1</td>
<td>26.2 (9.8)</td>
<td>0.76 (0.01)</td>
<td>0.05 (0.01)</td>
</tr>
<tr>
<td>G2</td>
<td>1031.0 (74.2)</td>
<td>0.90 (0.00)</td>
<td>0.06 (0.00)</td>
</tr>
<tr>
<td>G3</td>
<td>46.2 (13.7)</td>
<td>0.63 (0.14)</td>
<td>0.06 (0.01)</td>
</tr>
<tr>
<td>G4</td>
<td>924.3 (122.9)</td>
<td>0.87 (0.01)</td>
<td>0.09 (0.00)</td>
</tr>
<tr>
<td>A1</td>
<td>239.1 (20.6)</td>
<td>0.91 (0.01)</td>
<td>0.03 (0.00)</td>
</tr>
<tr>
<td>A2</td>
<td>219.5 (27.3)</td>
<td>1.00 (0.00)</td>
<td>0.02 (0.00)</td>
</tr>
<tr>
<td>A3</td>
<td>95.4 (10.8)</td>
<td>0.96 (0.00)</td>
<td>0.02 (0.00)</td>
</tr>
<tr>
<td>A4</td>
<td>146.3 (14.3)</td>
<td>0.91 (0.00)</td>
<td>0.06 (0.00)</td>
</tr>
</tbody>
</table>

$^a$ average over the incubation period
complicate further interpretation. The extent of O exchange during NO$_3^-$ reduction ($X_{ERR}$) was significant for all soils, ranging between 32% (G1) to almost 100% (A1, A2, A3; Table 4.3). The N$_2$O was mainly derived from NO$_3^-$-N (fertilizer-N) for most soils. On average $N_2O(NO_3)$ was 77.2%; for 4 out of the 10 soils it was more than 95% (so $N_2O(NH_4)$ less than 5%). In soil G3, N$_2$O was almost evenly derived from NO$_3^-$-N and NH$_4^+$-N, and only in soil G1 most N$_2$O-N originated from NH$_4^+$ (Table 4.3).

The partial OI and relative contributions to N$_2$O production of the different pathways are presented in Table 4.5, and the therewith calculated MOIs under all scenarios in Table 4.6. The ranges in the MOI obtained from the sensitivity analyses on the $X_{ERR}$ are included in Table 4.6. As a result of the high rates of O exchange quantified for denitrification ($X_{ERR}$), the pathways in general facilitated high rates of O incorporation (except for NN, as defined). In most soils the N$_2$O was mainly derived from NO$_3^-$, i.e. the contribution of fertilizer denitrification.

Table 4.5: The partial O incorporations ($OI_p$) and relative contributions to total N$_2$O production ($N_2O_p$) of the different pathways under the different scenarios A-F. The $OI_p$ and $N_2O_p$s are derived as described in appendices 4-1 and 4-2.

<table>
<thead>
<tr>
<th>Soil</th>
<th>$OI_{FD}$</th>
<th>$OI_{NCD}$</th>
<th>$OI_{IND}$</th>
<th>$OI_{NN}$</th>
<th>Pathway contributions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-F</td>
<td>A, B</td>
<td>C, D, E, F</td>
<td>A, C</td>
<td>B, E</td>
</tr>
<tr>
<td>F1</td>
<td>0.78</td>
<td>0.93</td>
<td>0.98</td>
<td>0.50</td>
<td>0.89</td>
</tr>
<tr>
<td>F2</td>
<td>0.89</td>
<td>0.96</td>
<td>1.00</td>
<td>0.50</td>
<td>0.94</td>
</tr>
<tr>
<td>G1</td>
<td>0.32</td>
<td>0.77</td>
<td>0.85</td>
<td>0.50</td>
<td>0.66</td>
</tr>
<tr>
<td>G2</td>
<td>0.90</td>
<td>0.97</td>
<td>1.00</td>
<td>0.50</td>
<td>0.95</td>
</tr>
<tr>
<td>G3</td>
<td>0.79</td>
<td>0.93</td>
<td>0.98</td>
<td>0.50</td>
<td>0.89</td>
</tr>
<tr>
<td>G4</td>
<td>0.82</td>
<td>0.94</td>
<td>0.99</td>
<td>0.50</td>
<td>0.91</td>
</tr>
<tr>
<td>A1</td>
<td>0.96</td>
<td>0.99</td>
<td>1.00</td>
<td>0.50</td>
<td>0.98</td>
</tr>
<tr>
<td>A2</td>
<td>0.97</td>
<td>0.99</td>
<td>1.00</td>
<td>0.50</td>
<td>0.98</td>
</tr>
<tr>
<td>A3</td>
<td>0.95</td>
<td>0.98</td>
<td>1.00</td>
<td>0.50</td>
<td>0.98</td>
</tr>
<tr>
<td>A4</td>
<td>0.87</td>
<td>0.96</td>
<td>0.99</td>
<td>0.50</td>
<td>0.94</td>
</tr>
</tbody>
</table>
(\(N_2O_{15} \)) was generally large (Table 4.5). In most soils the majority of the \(N_2O(NH_4)\) could theoretically be associated with the NCD pathway. In only three soils (G1, G3, A3) a contribution of direct \(N_2O\) production by nitrifiers (NN plus ND) of minimally 5% was assigned. As a result, the calculated MOIs were high for nearly all soils under all scenarios (Table 4.6). A comparison between the MOIs and AOI for the soils with a minimum nitrifier contribution of 10% is depicted in Figure 4.2a. Figure 4.2b presents the comparison of the AOI with the OI resulting from the evaluations S1 and S2, where the NCD contribution is not maximized. Differences between AOI and OI-S1 and OI-S2 (Figure 4.2b) were larger than those between AOI and MOI (Figure 4.2a), especially in those scenarios that include O exchange in fewer reaction steps (A,C).

Table 4.6: The maximum O incorporation (MOI, %) for scenario A-F on the presence of O exchange, constrained by the \(O_{18}P\) and \(N_2O_{15}P\). For soils where all \(NH_4^{+}\) derived \(N_2O\) may theoretically be ascribed to NCD, the MOI under B equals A, and D, E and F equal C. Results from the sensitivity analyses (sa) provide the range of the MOI for \(XERR \pm 10\%\).

<table>
<thead>
<tr>
<th>Soil</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>78.6</td>
<td>78.6</td>
<td>78.7</td>
<td>78.7</td>
<td>78.7</td>
<td>78.7</td>
</tr>
<tr>
<td>sa</td>
<td>86.3-70.9</td>
<td>86.3-70.9</td>
<td>86.4-71.0</td>
<td>86.4-71.0</td>
<td>86.4-71.0</td>
<td>86.4-71.0</td>
</tr>
<tr>
<td>F2</td>
<td>88.8</td>
<td>88.8</td>
<td>88.8</td>
<td>88.8</td>
<td>88.8</td>
<td>88.8</td>
</tr>
<tr>
<td>sa</td>
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<td>97.7-79.9</td>
<td>97.7-80.0</td>
<td>97.7-80.0</td>
<td>97.7-80.0</td>
<td>97.7-80.0</td>
</tr>
<tr>
<td>G1</td>
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<td>66.0</td>
<td>79.3</td>
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<tr>
<td>sa</td>
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<td>75.4-72.7</td>
<td>67.7-64.2</td>
<td>80.9-77.7</td>
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<td>90.6</td>
<td>90.6</td>
<td>90.6</td>
<td>90.6</td>
<td>90.6</td>
<td>90.6</td>
</tr>
<tr>
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<td>99.5-81.8</td>
<td>99.5-81.8</td>
<td>99.5-81.8</td>
<td>99.5-81.8</td>
</tr>
<tr>
<td>G3</td>
<td>81.2</td>
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<td>83.2</td>
<td>86.8</td>
<td>86.5</td>
<td>87.3</td>
</tr>
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<td>87.9-76.2</td>
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<td>91.6-81.2</td>
<td>92.1-82.2</td>
</tr>
<tr>
<td>G4</td>
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<td>82.7</td>
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<td>82.7</td>
</tr>
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<td>90.7-74.6</td>
<td>90.7-74.6</td>
<td>90.7-74.6</td>
<td>90.7-74.6</td>
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<td>A1</td>
<td>95.8</td>
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<td>96.1</td>
<td>96.1</td>
<td>96.1</td>
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<td>100-87.6</td>
<td>100-87.6</td>
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<td>A2</td>
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<td>97.3</td>
<td>96.1</td>
<td>97.5</td>
<td>97.5</td>
<td>97.5</td>
</tr>
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<td>100-89.9</td>
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</tr>
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<td>A3</td>
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<td>96.1</td>
<td>92.5</td>
<td>96.5</td>
<td>96.5</td>
<td>96.7</td>
</tr>
<tr>
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<td>100-88.4</td>
<td>95.8-85.7</td>
<td>100-89.5</td>
<td>100-89.3</td>
<td>100-89.8</td>
</tr>
<tr>
<td>A4</td>
<td>88.2</td>
<td>88.4</td>
<td>88.7</td>
<td>88.9</td>
<td>88.9</td>
<td>88.9</td>
</tr>
<tr>
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<td>96.3-81.0</td>
<td>96.5-81.2</td>
<td>96.5-81.2</td>
<td>96.5-81.3</td>
</tr>
</tbody>
</table>

sa: sensitivity analyses, \(XERR \pm 10\%\)
Discussion

Oxygen exchange during nitrifier-mediated N₂O production pathways

The measured O incorporation from H₂O into N₂O cannot for all soils be explained by O exchange during denitrification alone. The results therefore suggest that O exchange can also occur during nitrifier-mediated processes. In some of the soils, the contribution of the nitrifier pathways to total N₂O production was negligible. We therefore focus on those soils which showed a contribution of nitrifiers to N₂O production, either directly (NN and ND) and/or indirectly (NCD), of at least 10% (soils G1, G3, A1, A2, A3 and A4; Table 4.5).

Figure 4.2: Comparison of the actual (AOI) and maximum O incorporation for all scenario evaluations (MOI) for soils G1, G3, A1, A2, A3 and A4. In these soils minimally 10% of the N₂O was NH₄⁺-derived, i.e. an indirect (NCD) or direct (NN or ND) contribution of nitrifiers. Error bars indicate the SE of the AOI. (a) The AOI and the MOI for all scenarios. For the calculation of the MOI, the N₂ONCD is maximized. (b) The AOI and the S1-OI and S2-OI for all scenarios. Under S1 and S2, N₂OND and N₂Orev are respectively maximized. For S1, the OI under C will equal A, and E will equal B. For S2, the OI equals for all scenarios.
Our results showed that O exchange between H\textsubscript{2}O and intermediates of N\textsubscript{2}O production occurred in nitrifier pathways in at least two out of the six above mentioned soils. For these soils, G1 and A3, comparison of the AOI and MOI for the different scenarios implied that O exchange must have been present in nitrifier pathways to reach the measured AOI. For the pathway of nitrifier denitrification (ND, Figure 4.1(ii)), the analyses of both soils confirmed the presence of O exchange because the AOI is in better agreement with the MOI when it is included in the scenarios (scenario B, E, and F vs. A, C, and D, respectively). We anticipated that the presence of O exchange in nitrifier denitrification would be likely since the enzymes involved in N\textsubscript{2}O production of that pathway are similar to the denitrifier enzymes (Casciotti et al., 2001; Chain et al., 2003; Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007). Moreover, several monoculture studies with denitrifiers have identified O exchange during reduction of NO\textsubscript{2} (Garber et al., 1982; Aerssens et al., 1986; Shearer et al., 1988; Ye et al., 1991). Next to the ND pathway, results also supported the notion that O exchange may take place during NO\textsubscript{2} conversion to NO\textsubscript{3} by nitrite oxidizers, affecting the O isotopic composition of both NO\textsubscript{2} and NO\textsubscript{3} (Figure 4.1, iii-b). In soil G1, comparison of scenarios D with A and F with B showed that the AOI was better explained when such O exchange was assumed to be present (Figure 4.2a, Table 4.6). Oxygen exchange during NO\textsubscript{2} conversion to NO\textsubscript{3} without affecting the NO\textsubscript{2}-O (Figure 4.1, iii-a) would not provide sufficient additional O incorporation to explain the observed AOI (scenario C versus A).

Apart from the large relative contribution of FD to total N\textsubscript{2}O production, our evaluation of O exchange during nitrifier pathways is complicated by two main (required) assumptions: i.e. the assumptions on the contribution of NCD to N\textsubscript{2}O and on the extent of O exchange. Due to the large allocated N\textsubscript{2}ONCD and XERR, the calculated MOIs are high and vary only marginally for the different scenarios. However, for both assumptions it holds that this evaluation likely underestimates the presence of O exchange, and that O exchange in N\textsubscript{2}O production pathways by nitrifiers in reality would be more profound, as discussed below.

A smaller percentage of NH\textsubscript{4}\textsuperscript{+} derived N\textsubscript{2}O assigned to NCD would leave a larger contribution to NN plus ND. The pathways of NN and ND allow less O incorporation from H\textsubscript{2}O through reaction stoichiometry, so the maximization of NCD implies that the MOI we calculate may overestimate the stoichiometric OI.
It would then jointly underestimate the OI through O exchange, i.e. a larger part of the AOI would in fact be due to O exchange. When ND or NN were maximized (Figure 4.2b) at the expense of NCD (S1 and S2 respectively), analyses of soil G1 and A3 further support the presence of O exchange during nitrifier pathways as already confirmed with the MOI. In three of the other four soils that showed a minimal nitrifier contribution of 10% (A1, A2 and A4), the majority of the $N_2O_{(NH)}$ could theoretically be ascribed to NCD. Under S1 and S2, all three showed that additional O exchange by nitrifiers would be required to explain the AOI when ND or NN were maximized instead of NCD. Of all soils, the effect of maximizing the NCD contribution was most prominent for soil G3. In this single soil, the AOI did not exceed the MOI, not even when O exchange was considered to be present only during denitrification (scenario A). However, when all $N_2O$ would have been produced through NN and FD (S2), the AOI did exceed S2-OI. Hence, these results indicate that, also in soil G3, O exchange may have occurred during nitrifier pathways in addition to O exchange during denitrification. Here it remains uncertain whether the measured AOI is partially the result of nitrifier-mediated O exchange, or solely the result of O incorporation through reaction stoichiometry plus O exchange through denitrifiers. Unfortunately, the evaluation of the results of this soil (G3) was also complicated by the relatively large variation in the measured AOI.

Our assumption on the extent of O exchange may also complicate the evaluation. We assume that wherever O exchange might occur, it takes place at the same rate as quantified for denitrification of NO$_3^-$ to N$_2$O. Fortunately, the results of our sensitivity analyses showed that a variation of 10% in the $X_{ERR}$ would not result in large changes in the MOIs. However, it is likely that the extent of exchange in separate reaction steps is smaller than $X_{ERR}$, as the total exchange during NO$_3^-$ reduction to N$_2$O can be a sum of exchange in separate reaction steps. The ND pathway does not include the NO$_3^-$ to NO$_2^-$ reduction step (Figure 4.1, ii) and NO$_2^-$ oxidation comprises only the conversion of NO$_2^-$ to NO$_3^-$ (Figure 4.1 iii). A lower level of exchange in separate reaction processes would result in lower MOIs. Oxygen exchange may then be required in both the ND pathway (Figure 4.1, ii) and the nitrite oxidation step (Figure 4.1 iii) to explain the observed AOI. In conclusion, if our $X_{ERR}$ overestimates the extent of exchange per process, O exchange would in fact be more substantial for the nitrifier pathways.
Consideration of other N\textsubscript{2}O production pathways

In our analyses we considered nitrification, denitrification, and nitrifier denitrification as major N\textsubscript{2}O production processes. However, N\textsubscript{2}O may also evolve from dissimilatory nitrate reduction to ammonia (DNRA) (Stevens et al., 1998), aerobic denitrification (Lloyd et al., 1987; Bell et al., 1991; Patureau et al., 2000; Bateman et al., 2005), fungal denitrification (Bollag et al., 1972; Shoun et al., 1992) and co-denitrification (Garber et al., 1982; Tanimoto et al., 1992b; Laughlin et al., 2002; Morozkina et al., 2007).

Regarding DNRA, the intermediate levels of anaerobicity made its occurrence in our experiment unlikely, and insignificant $^{15}$N enrichment of the NH\textsubscript{4}\textsuperscript{+} after application of enriched NO\textsubscript{3}\textsuperscript{-} showed that N\textsubscript{2}O production through this pathway was indeed negligible in all soils. Consideration of fungal and aerobic denitrification would not change the outcome of our evaluation based on isotope tracing; their contribution is included in the fertilizer and nitrification-coupled denitrification (FD and NCD), regardless of whether the process is carried out by fungi or bacteria, or under aerobic or anaerobic conditions. However, in co-denitrification part of the denitrification-derived N\textsubscript{2}O may have been obtained from another N source than fertilizer- or nitrification-derived NO\textsubscript{3}\textsuperscript{-}. This would dilute the anticipated $^{15}$N enrichment in TR3 and thus cause an underestimation of the extent of O exchange as quantified by the $X$\textsubscript{ERR} (Kool et al., 2009a). If part of the N\textsubscript{2}O was produced by co-denitrification, it would also alter the allocation of N\textsubscript{2}O production across the different pathways, i.e. the contribution of the nitrifier pathways would be overestimated. This could complicate the identification of the presence of O exchange during production processes by nitrifiers. The relative low $^{15}$N-N\textsubscript{2}O enrichment compared to that of the applied $^{15}$N-NO\textsubscript{3}\textsuperscript{-} may be indicative of the presence of another N source. However, the same is seen for the $^{18}$O enrichment, where the discrepancy is even greater as depicted by the ERR. Moreover, occurrence of co-denitrification would result in an underestimation of the O exchange as quantified by our ERR approach, while the O exchange quantified here as such is high already. We therefore consider it unlikely that co-denitrification comprised a significant contribution to N\textsubscript{2}O production from the soils in our incubation.
Conclusion

We showed that O exchange between H₂O and intermediates of N₂O production can affect the origin of O in N₂O from both nitrifier and denitrifier pathways. For denitrification, O exchange was confirmed to be present for all soils. For nitrifier pathways, results from two of the six soils that showed a minimum nitrifier contribution to N₂O production of 10% proved that O exchange can occur during nitrifier pathways. For three out of these six, based on our sub-evaluation, we suggest that it is very likely that nitrifier production pathways were accompanied by O exchange as well. Moreover, also for the last of the six soils showing a minimum nitrifier contribution to N₂O production of 10%, our evaluation showed that O exchange may not have been limited to denitrification.

Although it may be a less widespread feature across different soils than demonstrated for denitrification, we conclude that O exchange can indeed occur during nitrifier pathways. We therefore advocate that the O isotopic signature of N₂O should be interpreted with caution when used to derive information on the origin of N₂O. Oxygen isotopic analyses of N₂O can still be a useful tool to derive information on the origin of N₂O, but based on our results we conclude that the previously proposed approach by Wrage et al. (2005) does not suffice to distinguish completely between the targeted production pathways. For both nitrifier and denitrifier pathways, future studies are needed to signify the importance of O exchange between H₂O and intermediates of N₂O production in soil. This will ultimately lead to an improved process-based understanding of different pathways of N₂O production in terrestrial ecosystems.

Acknowledgements

This research was financed by the NitroEurope IP (GOCE-017841), funded by the European Commission under the 6th framework program. J.W. Van Groenigen is supported by a personal VIDI grant from the Netherlands Organization of Scientific Research/Earth and Life Sciences (NWO-ALW).
Appendices

Appendix 4-1: Partial H\textsubscript{2}O-O incorporation of the N\textsubscript{2}O production pathways

The calculated partial H\textsubscript{2}O-O incorporation of the pathways $OI(N_2O)$s under the different scenarios A-F are listed in Table 4.5. Below we provide a detailed description of their derivation.

4-1.1: Denitrifier N\textsubscript{2}O production (FD & NCD)

For both denitrifier pathways (FD and NCD) and under all scenarios, the N\textsubscript{2}O produced has been subject to exchange of O between H\textsubscript{2}O and intermediate compounds of NO\textsubscript{3}\textsuperscript{-} reduction to N\textsubscript{2}O, as expressed by the $X_{ERR}$. The fraction of the O in N\textsubscript{2}O originating from H\textsubscript{2}O will further depend on the O incorporated from H\textsubscript{2}O into NO\textsubscript{3}\textsuperscript{-} ($OI(NO_3^p)$), which does differ for the pathways and scenarios. For N\textsubscript{2}O produced through FD and NCD holds:

$$OI(N_2O_{FD}) = OI(NO_3_{FD}) + X_{ERR} \cdot (1 - OI(NO_3_{FD})) \quad [A,B,C,D,E,F]$$

$$OI(N_2O_{NCD}) = OI(NO_3_{NCD}) + X_{ERR} \cdot (1 - OI(NO_3_{NCD})) \quad [A,B,C,D,E,F]$$

In case of FD, applied NO\textsubscript{3}\textsuperscript{-} will have no O incorporated from the enriched H\textsubscript{2}O through reaction stoichiometry during incubation, i.e. the $OI(NO_3_{FD})$ is zero. As a result:

$$OI(N_2O_{FD}) = X_{ERR} \quad [A,B,C,D,E,F]$$

For NCD, the nitrification-derived NO\textsubscript{3}\textsuperscript{-} will have obtained 2/3\textsuperscript{rd} of the O from H\textsubscript{2}O according to reaction stoichiometry (Figure 4.1). When O exchange occurs during nitrification of NO\textsubscript{2}\textsuperscript{-} to NO\textsubscript{3}\textsuperscript{-} (scenario C, D, E, F), additional O will be incorporated from H\textsubscript{2}O into the nitrification-derived NO\textsubscript{3}\textsuperscript{-}:

$$OI(NO_3_{NCD}) = 2/3 \quad [A,B]$$

$$OI(NO_3_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 \quad [C,D,E,F]$$

In the N\textsubscript{2}O produced from NO\textsubscript{3}\textsuperscript{-} through NCD, the $OI(N_2O_{NCD})$ thus amounts to:
Oxygen exchange between nitrogen oxides and H2O during nitrifier pathways

\begin{align*}
    OI(N_2O_{NCD}) &= \frac{2}{3} + X_{ERR} \cdot \frac{1}{3} \quad [A,B] \\
    OI(N_2O_{NCD}) &= \frac{2}{3} + X_{ERR} \cdot \frac{1}{3} + X_{ERR} \cdot (1-(\frac{2}{3} + X_{ERR} \cdot \frac{1}{3})) \quad [C,D,E,F]
\end{align*}

A4.1.2: Nitrifier N2O production (ND & NN)

No oxygen will be incorporated from H2O in the N2O produced directly through nitrifiers as a by-product of nitrification, N2O_{NN}, through reaction stoichiometry or through O exchange, in any of the scenarios:

\[ OI(N_2O_{NN}) = 0 \quad [A,B,C,D,E,F] \]

The OI(N2O_p) for production by nitrifiers through ND (OI(N2O_{ND})) differs for the O exchange scenarios. When no O exchange takes place during NO2- reduction (A,C,D), the OI(N2O_{ND}) will remain the same as the fraction of the O originating from H2O in the preceding NO2- (OI(NO2)). Under scenario B, E, and F, the O exchange during NO2- reduction to N2O adds to the OI(N2O_{ND}):

\[ OI(N_2O_{ND}) = OI(NO2) \quad [A,C,D] \]
\[ OI(N_2O_{ND}) = OI(NO2) + X_{ERR} \cdot (1-OI(NO2)) \quad [B,E,F] \]

The OI(NO2) also differs for the various O exchange scenarios. The NO2- will have obtained 50% of its O from H2O, following reaction stoichiometry of its production (Figure 4.1). When O exchange is assumed to affect the NO2- (D, F; Figure 4.1 iiib), the OI(NO2) will be identical to OI(NO3_{NCD}):

\[ OI(NO2) = \frac{1}{2} \quad [A,B,C,E] \]
\[ OI(NO2) = \frac{2}{3} + X_{ERR} \cdot \frac{1}{3} \quad [D,F] \]

The OI for N2O produced directly through ND (OI(N2O_{ND})) thus becomes:

\[ OI(N_2O_{ND}) = \frac{1}{2} \quad [A,C] \]
\[ OI(N_2O_{ND}) = \frac{1}{2} + X_{ERR} \cdot \frac{1}{2} \quad [B,E] \]
\[ OI(N_2O_{ND}) = \frac{2}{3} + X_{ERR} \cdot \frac{1}{3} \quad [D] \]
\[ OI(N_2O_{ND}) = \frac{2}{3} + X_{ERR} \cdot \frac{1}{3} + X_{ERR} \cdot (1-(\frac{2}{3} + X_{ERR} \cdot \frac{1}{3})) \quad [F] \]
In summary, the $OI(N_2O_p)$s for all pathways are calculated as follows:

$$OI(N_2O_{FD}) = X_{ERR} \quad [A,B,C,D,E,F]$$

$$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 \quad [A,B]$$

$$OI(N_2O_{NCD}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3)) \quad [C,D,E,F]$$

$$OI(N_2O_{ND}) = 1/2 \quad [A,C]$$

$$OI(N_2O_{ND}) = 1/2 + X_{ERR} \cdot 1/2 \quad [B,E]$$

$$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3 \quad [D]$$

$$OI(N_2O_{ND}) = 2/3 + X_{ERR} \cdot 1/3 + X_{ERR} \cdot (1-(2/3 + X_{ERR} \cdot 1/3)) \quad [F]$$

$$OI(N_2O_{NN}) = 0 \quad [A,B,C,D,E,F]$$

**Appendix 4-2: Relative contribution of the $N_2O$ production pathways**

The different pathways facilitate different amounts of $H_2O-O$ incorporation through reaction stoichiometry. To calculate the theoretical maximum oxygen incorporation (MOI), the contribution of the pathways that facilitate highest $H_2O-O$ incorporation through reaction stoichiometry is maximized. In line with eq 4.6:

$$MOI = \sum N_2O_p \cdot OI(N_2O_p),$$

while maximizing the $N_2O_p$s associated with the largest $OI(N_2O_p)$ (eq A4.1)

The $N_2O_p$s are constrained by the variables calculated from the incubation results. This way, the stoichiometric $O$ incorporation is maximized, which ensures we never overestimate the presence of $O$ exchange.

Briefly, the relative contribution of fertilizer denitrification ($N_2O_{FD}$) follows directly from the treatments where $^{15}N$ enriched mineral $N$ was applied. To maximize the stoichiometric $O$ incorporation from $H_2O$ (Figure 4.1), subsequently the $N_2O_{NCD}$ and $N_2O_{ND}$ are maximized. The $N_2O_p$s are presented in Table 4.5 for all soils.

**A4-2.1: Denitrifier $N_2O$ production (FD & NCD)**

$N_2O_{(NO3)}$ represents the contribution of fertilizer denitrification (FD) to total $N_2O$ production, $N_2O_{FD}$ (%). The $N_2O_{(NH4)}$ comprises the relative contribution to total...
Oxygen exchange between nitrogen oxides and H₂O during nitrifier pathways

N₂O production (%) of the nitrifier nitrification (NN; N₂O_NN), nitrifier denitrification (ND; N₂O_ND), plus nitrification-coupled denitrification (NCD; N₂O_NCD) pathways:

\[
N₂O_{NO₃} = N₂O_{FD} \quad \text{(eq A4.2)}
\]

\[
N₂O_{NH₄} = N₂O_{NN} + N₂O_{ND} + N₂O_{NCD} \quad \text{(eq A4.3)}
\]

For further distinction between the relative contribution of the NN, ND and NCD pathways, we evaluate the ¹⁵N enrichment of the N₂O and NO₃⁻ resulting from application of ¹⁵N enriched NH₄⁺ (TR4). The relative contribution of these pathways to the total N₂O(NH₄) cannot be exactly calculated, so to maximize stoichiometric H₂O-O incorporation we first derive the maximal possible contribution of NCD. As long as the ¹⁵N enrichment in the total N₂O does not exceed the ¹⁵N enrichment of the NO₃⁻ during the incubation (with TR4, application of ¹⁵N enriched NH₄⁺), the N₂O_{NH₄} may have exclusively originated from NCD. So all N₂O_{NH₄} is then ascribed to N₂O_{NCD}, and N₂O_{NN} and N₂O_{ND} are assumed to be zero:

If \( ¹⁵N(N₂O_{TR4}) \leq ¹⁵N(NO₃⁻_{TR4}) \):

\[
N₂O_{FD} = N₂O_{NO₃}
\]

\[
N₂O_{NCD} = N₂O_{NH₄}
\]

\[
N₂O_{ND} = 0
\]

\[
N₂O_{NN} = 0
\]

A4-2.2: Nitrifier N₂O production (ND & NN)

When the ¹⁵N-N₂O exceeded the ¹⁵N enrichment in the NO₃⁻ in TR4, i.e. when \( ¹⁵N(N₂O_{TR4}) > ¹⁵N(NO₃⁻_{TR4}) \), not all NH₄⁺-N that ended up in N₂O had gone through the nitrification-coupled denitrification. In other words, a minimal contribution to N₂O production by nitrifiers through NN or ND (N₂O_NN plus N₂O_ND; N₂O_{NN+ND}) must be adopted. The ¹⁵N-N₂O and ¹⁵N-NO₃⁻ enrichment in TR4 is then used to provide information on the ratio of N₂O production by NCD versus NN plus ND. This ratio of N₂O_{NCD} versus N₂O_{NN+ND} reflects the ratio of ¹⁵N enrichments of these pools, \( ¹⁵N(N₂O_{NCD}) \) and \( ¹⁵N(N₂O_{NN+ND}) \). These in turn will
Chapter 4

reflect the N isotopic signature of their N₂O preceding compounds NO₃⁻ and NH₄⁺, \(^{15}\text{N}(\text{NO}_3^-_{(TR3)})\) and \(^{15}\text{N}(\text{NH}_4^+_{(TR4)})\), respectively:

\[
\frac{N_2O_{NCD}}{N_2O_{NN+ND}} = \frac{^{15}\text{N}(N_2O_{NCD})}{^{15}\text{N}(N_2O_{NN+ND})} = \frac{^{15}\text{N}(\text{NO}_3^-_{(TR4)})}{^{15}\text{N}(\text{NH}_4^+_{(TR4)})}
\]

(\text{eq A4.4})

Combined with \text{eq A4.3} it follows:

\[
N_2O_{(NH4)} = N_2O_{NCD} + N_2O_{NCD} \cdot \left( \frac{^{15}\text{N}(\text{NH}_4^+_{(TR4)})}{^{15}\text{N}(\text{NO}_3^-_{(TR4)})} \right), \text{ i.e.}
\]

\[
N_2O_{(NH4)} = N_2O_{NCD} \cdot \left( 1 + \frac{^{15}\text{N}(\text{NH}_4^+_{(TR4)})}{^{15}\text{N}(\text{NO}_3^-_{(TR4)})} \right), \text{ i.e.}
\]

\[
N_2O_{(NH4)} = N_2O_{NCD} \cdot \left( \frac{^{15}\text{N}(\text{NO}_3^-_{(TR4)}) + ^{15}\text{N}(\text{NH}_4^+_{(TR4)})}{^{15}\text{N}(\text{NO}_3^-_{(TR4)})} \right), \text{ i.e.}
\]

\[
N_2O_{NCD}=N_2O_{(NH4)} \left( \frac{^{15}\text{N}(\text{NO}_3^-_{(TR4)}) + ^{15}\text{N}(\text{NH}_4^+_{(TR4)})}{^{15}\text{N}(\text{NO}_3^-_{(TR4)})} \right)
\]

(\text{eq A4.5})

To maximize stoichiometric OI, the remaining portion of \(N_2O_{(NH4)}\), which constitutes \(N_2O_{NN}\) plus \(N_2O_{ND}\), is all ascribed to ND. The pathway of NN is assumed not to contribute to any O incorporation from H₂O into N₂O, and therefore set to zero. In summary, the N₂O assigned to the different pathways amounts to:

\text{If} \quad ^{15}\text{N}(N_2O_{(TR4)}) > ^{15}\text{N}(\text{NO}_3^-_{(TR4)}):\n
\[
N_2O_{FD} = N_2O_{(NO3)}
\]

\[
N_2O_{NCD} = N_2O_{(NH4)} \cdot \left( \frac{^{15}\text{N}(\text{NO}_3^-_{TR4}) + ^{15}\text{N}(\text{NH}_4^+_{TR4})}{^{15}\text{N}(\text{NO}_3^-_{TR4}) + ^{15}\text{N}(\text{NH}_4^+_{TR4})} \right)
\]

(\text{eq A4.5})

\[
N_2O_{ND} = N_2O_{(NH4)} - N_2O_{NCD}
\]

\[
N_2O_{NN} = 0
\]
**A4-2.3: Sub-evaluation: maximizing direct nitrifier N₂O production (NN & ND)**

Our evaluation of the MOI requires the maximization of the contribution of NCD. In reality its contribution may however be smaller. We therefore derive additional sets of OIs for two cases, S1 and S2, where the NH₄⁺ derived N₂O is assigned to NCD, ND and NN differently. All N₂O(NH₄) is ascribed to either ND (S1) or to NN (S2), i.e. the N₂O_{NCD} is zero:

**S1-OI:**
\[ \text{N}_2\text{O}_{FD} = N_2O_{(NO_3)} \]
\[ N_2O_{NCD} = 0 \]
\[ N_2O_{ND} = N_2O_{(NH_4)} \]
\[ N_2O_{NN} = 0 \]

**S2-OI:**
\[ \text{N}_2\text{O}_{FD} = N_2O_{(NO_3)} \]
\[ N_2O_{NCD} = 0 \]
\[ N_2O_{ND} = 0 \]
\[ N_2O_{NN} = N_2O_{(NH_4)} \]

Note that as the N₂O_{NCD} is zero, the OI resulting from these sub-evaluations under C will equal A, and E will equal B. Under S2 the N₂O_{ND} is zero as well, and as the OI(N₂O_{NN}) is zero under all scenarios, the total S2-OI will only depend on the FD contribution and its partial OI, and thus be the same for scenario A to F.
Nitrifier denitrification can be a source of N$_2$O from soil: a revised approach to the dual isotope labeling method

Abstract  
Nitrifier denitrification, i.e. nitrite reduction by ammonia oxidizers, is one of the biochemical pathways of nitrous oxide (N$_2$O) production. It is increasingly suggested that this pathway may contribute substantially to N$_2$O production in soil, the major source of this greenhouse gas. However, although monoculture studies recognize its potential, methodological drawbacks prohibit conclusive proof that nitrifier denitrification occurs in actual soils. Here we suggest and apply a new isotopic approach to identify its presence in soil. In incubation experiments with twelve soils, N$_2$O production was studied using oxygen (O) and nitrogen (N) isotope tracing, accounting for O exchange. Microbial biomass C and N and phospholipid fatty acid (PLFA) patterns were analyzed to explain potential differences in N$_2$O production pathways. We found that in at least five of the soils, nitrifier denitrification must have contributed to N$_2$O production. Moreover, it may even have been responsible for all NH$_4^+$ derived N$_2$O in most soils. In contrast, N$_2$O as a by-product of ammonia oxidation contributed very little to total production. Microbial biomass C and N and PLFA-distinguished microbial community composition were not indicative of differences in N$_2$O production pathways. Overall, we show that combined O and N isotope tracing may still provide a powerful tool to understand N$_2$O production pathways, provided that O exchange is accounted for. We conclude that nitrifier denitrification can indeed occur in soils, and may in fact be responsible for the majority of total nitrifier-induced N$_2$O production.

Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas and contributes to the breakdown of stratospheric ozone (Crutzen, 1981). Globally, soils constitute the major source of N\textsubscript{2}O to the atmosphere (IPCC, 2007). Rising atmospheric N\textsubscript{2}O concentrations over the last decades have led to increased interest in understanding the production pathways of N\textsubscript{2}O, in order to enable development of adequate mitigation strategies.

Traditionally, autotrophic nitrification and heterotrophic denitrification have been considered to be the major N\textsubscript{2}O forming processes. However, it has long been acknowledged that these are not the sole production pathways of N\textsubscript{2}O. Nitrifier denitrification (denitrification by autotrophic nitrifiers) (Hooper, 1968; Ritchie et al., 1972), heterotrophic nitrification (Verstraete et al., 1973; Papen et al., 1989; Laughlin et al., 2008) and co-denitrification (Shoun et al., 1991; Tanimoto et al., 1992b; Laughlin et al., 2002) by both fungi and bacteria, as well as dissimilatory nitrate reduction to ammonia (DNRA) (Caskey et al., 1979; Smith et al., 1981; Bleakley et al., 1982) may all produce N\textsubscript{2}O as (by-) product. For most of these processes the relative significance for N\textsubscript{2}O production was long thought to be minor in soils compared to nitrification (NN) and denitrification (from fertilizer, FD, or coupled with nitrification, NCD) (Figure 5.1). However, for nitrifier denitrification (ND) it is increasingly suggested that it may constitute a considerable contribution to N\textsubscript{2}O production in soil (Webster et al., 1996; Wrage et al., 2004a; Ma et al., 2007; Sánchez-Martín et al., 2008).

In pure cultures the existence of nitrifier denitrification has long been established (Hooper, 1968; Ritchie et al., 1972). Several ammonia-oxidizing bacteria (AOB) have since then been identified to be able to denitrify nitrite (NO\textsubscript{2}\textsuperscript{-}) to N\textsubscript{2}O (Poth et al., 1985; Remde et al., 1990; Zart et al., 1998; Colliver et al., 2000; Schmidt et al., 2004b; Shaw et al., 2006). \textit{Nitrosomonas europaea} has been studied most extensively, but is less representative of the microbial community commonly found in soils (Kowalchuk et al., 2001; Wrage et al., 2001; Arp et al., 2003; Shaw et al., 2006). The AOB most commonly found in soils are \textit{Nitrosospira} spp. (Stephen et al., 1996; Stephen et al., 1998; Kowalchuk et al., 2001; Smith et al., 2001). Wrage et al. (2004b) first suggested that N\textsubscript{2}O production by a representative of this genus (\textit{Nitrosospira briensis}) occurred partly through ND.
Nitrifier denitrification can be a source of N₂O from soil

Shaw et al. (2006) found that all their seven *Nitrosospira* spp. strains tested could indeed produce N₂O through ND in pure cultures, and suggested that the ability to denitrify may be a common trait among AOB. Moreover, the recently presented complete genome sequence of *Nitrosospira multiformis* revealed that, similar to *Nitrosomonas europaea*, it contains orthologs to copper-containing nitrite-reductase (*nirK*) and nitric oxide reductase (*norCBQD*), and no coding sequence with similarity to (known) nitrate or nitrous oxide reductases (Norton et al., 2008). However, Dundee and Hopkins (Dundee et al., 2001) suggested that observed differences in O₂ sensitivities with respect to N₂O production between *Nitrosomonas europaea* and *Nitrosolobus multiformis* (currently classified as *Nitrosospira* spp. (Head et al., 1993)) implied differences in their ability to produce N₂O through nitrifier denitrification (Dundee et al., 2001). Therefore, even if the ability for ND is a common trait among nitrifiers in pure cultures, the actual occurrence in soil and level of significance remains unclear.

Although soil-based studies increasingly propose that ND may be contributing to N₂O emission from soils (Webster et al., 1996; Hüttsch et al., 1999; Wrage et al., 2004a; McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martín et al., 2008), conclusive proof of its presence in soil remains elusive due to the lack of reliable analytical methodology. Earlier approaches were shown to entail various important drawbacks. The use of oxygen suppression and
acetylene inhibition (Yoshinari et al., 1977; Robertson et al., 1987; Klemedtsson et al., 1988; Webster et al., 1996) was shown to be unreliable (Tilsner et al., 2003; Beaumont et al., 2004b; Beaumont et al., 2004a; Wraga et al., 2004a; Wraga et al., 2004b; Shaw et al., 2006). $^{15}$N isotopic labeling techniques have been employed to differentiate and quantify N$_2$O production from denitrification and nitrification in soil (Stevens et al., 1997; Baggs et al., 2003; Tilsner et al., 2003; Bateman et al., 2005) but it does not distinguish the N$_2$O that results from nitrite reduction (i.e. nitrifier denitrification; ND) from the N$_2$O generated as by-product from ammonia oxidation (i.e. nitrifier nitrification; NN) (Wraga et al., 2005). To enable this further distinction a dual-isotope approach was proposed (Wraga et al., 2005) that combined $^{15}$N labeling with the use of $^{18}$O labeled water. However, it was recognized that potential O exchange between H$_2$O and intermediate compounds of N$_2$O production complicates data interpretation for this method (Wraga et al., 2005; Kool et al., 2007; Kool et al., 2009b; Kool et al., 2009a). Introducing an additional treatment with $^{18}$O labeled NO$_3^-$, Kool et al. (2009a) were able to quantify O exchange during denitrification. We here propose that these insights allow to partially account for O exchange and with a revised approach will allow to confirm whether ND occurs in soils, and to quantify margins of its relative contribution.

The aim of this study was thus to evaluate the N and O isotopic data from soil incubations with the revised dual isotope approach to assess whether N$_2$O production through ND may be conclusively proven. Further, we aim to investigate whether these results may reflect differences in soil characteristics and/or in the soil microbial community.

**Methods**

**Soil sampling**

Soil samples were collected from 12 sites across Europe, encompassing forest (F), grassland (G), and arable (A) fields (Table 5.1). For the soil incubation (isotope tracing) experiment, samples (0-10 cm) were dried at 40°C, sieved (2mm) and stored at 4°C until further use. Analyses for microbial biomass and community composition analyses were carried out on fresh soil samples (0-5cm). All soils were sampled simultaneously for the isotope tracing experiment and the
Nitrifier denitrification can be a source of N₂O from soil. Table 5.1. Soil properties, microbial biomass N and C, and microbial biomass PLFA of all soils (except G3). The soils originated from forest (F), grassland (G), and arable (A) sites across Europe.

| Soil (H₂O) mgC g⁻¹ dm | pH | C/N | Corg | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N | C/N |
|------------------------|----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| H2                     | 2.9 (1.0) | 2.9 (0.5) | 2.7 (0.5) | 2.5 (0.4) | 2.4 (0.2) | 2.3 (0.3) | 2.2 (0.2) | 2.1 (0.1) | 2.0 (0.0) | 1.9 (0.0) | 1.8 (0.0) | 1.7 (0.0) | 1.6 (0.0) | 1.5 (0.0) | 1.4 (0.0) | 1.3 (0.0) | 1.2 (0.0) | 1.1 (0.0) | 1.0 (0.0) | 0.9 (0.0) | 0.8 (0.0) | 0.7 (0.0) | 0.6 (0.0) | 0.5 (0.0) | 0.4 (0.0) | 0.3 (0.0) | 0.2 (0.0) | 0.1 (0.0) |

Values in brackets denote the standard error of the mean.

PH measured in CaCl₂

Table 5.1. Soil properties, microbial biomass N and C, and microbial biomass PLFA of all soils (except G3).
Chapter 5

microbial analysis, except for soil G4. Due to logistic complications, soil G3 was excluded from the soil microbial analyses.

Soil incubation experiment

Details of the incubation experiment were described by Kool et al. (2009a). In brief, for each treatment five replicate samples (75 g soil) were pre-incubated at 16°C and 40% water holding capacity (WHC) a week prior to the incubation. At the start of the incubation, all samples received equal amounts of mineral N (50 mg NH₄⁺-N kg⁻¹ and 50 mg NO₃⁻-N kg⁻¹ soil). They were incubated at 80% WHC by adding appropriate amounts of H₂O. The samples were treated with one of four combinations of ¹⁸O and ¹⁵N labeled compounds; ¹⁸O enriched H₂O at 1.0 atom% excess (TR1), ¹⁸O enriched NO₃⁻ at 1.0 atom% excess (TR2), ¹⁵N enriched NO₃⁻ at 40.0 atom% excess (TR3), and ¹⁵N enriched NH₄⁺ at 40.0 atom% excess (TR4). The experiment was set up as a completely randomized design. The sample jars were closed by lids equipped with rubber septa. At the end of the 28 h incubation period, gas and soil samples were taken. Gas samples were extracted from the headspace of the jars and transferred to 12ml exetainer vials. The N₂O concentration and its isotopic signature were measured at the UC Davis Stable Isotope Facility, using a Sercon Cryoprep trace gas concentration system interfaced to a Sercon 20/20 isotope ratio mass spectrometer (Sercon Ltd., Crewe, Cheshire, UK).

Soil samples were taken after gas sampling. The exact soil moisture content was determined from one set of sub-samples, other sub-samples of approximately 20 g moist soil were taken for analyses of mineral N (NH₄⁺-N and NO₃⁻-N) after extraction with 1M KCl (50 ml per 20g soil) followed by segmented flow analysis (Skalar Analytical, Breda, The Netherlands) (Kool et al., 2006). The ¹⁵N enrichments of the mineral N were derived using a microdiffusion method as described in Kool et al. (2009b).

Soil microbial analyses

Four replicate samples from each site were analyzed for several microbial parameters. Microbial biomass N was determined as ninhydrin-reactive N by chloroform fumigation-extraction and calculated as ninhydrin-reactive N times
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3.1 (Hackl et al., 2000). Microbial biomass C was determined by chloroform fumigation followed by DOC analysis of extracts by dry combustion (Schinner et al., 1995). Phospholipid fatty acid (PLFA) analyses were carried out as described by Hackl et al. (2005) to profile the microbial community composition. The total amount of PLFAs was taken to represent total microbial biomass. Specific groups of PLFAs were considered as indicators of bacterial (i14:0, a15:0, i15:0, i16:0, i17:0, a17:0, 10Me16:0, 10Me17:0, cy17:0, cy18:0, cy19:0, 16:1(9), 18:1(13), 18:1(11)), fungal (18:2(9,12)), actinomycete (10Me18:0) and vesicular arbuscular mycorrhizal (VAM) (16:1(11)) biomass (nmol g⁻¹ soil (dry matter)).

Data analysis: N2O production

Total N2O production, as well as isotopic enrichment of the N2O and soil mineral N pools from our soil incubation experiment have been reported in Kool et al. (2009b). From the 15N-tracing data we obtained the proportions of NH4⁺ and NO3⁻ derived N2O (N2O(NH4) and N2O(NO3), respectively), and the relative pathway contribution of fertilizer denitrification (FD) and the (theoretical) maximum fraction of nitrification-coupled denitrification (NCD) (N2OFD and N2ONCD-max, respectively). In appendix 5-1 (equations eq A5.1 through eq A5.7), we added a short description of the derivation of these parameters.

From the measured N2O production and the N2O(NH4) and N2O(NO3) proportions (%), we calculated the absolute NH4⁺ and NO3⁻ derived N2O production over the incubation period as well:

\[
\left[ N_2O(NH4) \right] = \left[ N_2O \right] \cdot \frac{N_2O(NH4)}{100}, \quad \text{and} \\
\left[ N_2O(NO3) \right] = \left[ N_2O \right] \cdot \frac{N_2O(NO3)}{100}
\]

(eq 5.1)

(eq 5.2)

where \([N_2O]\), \([N_2O(NH4)]\) and \([N_2O(NO3)]\) represent the absolute N2O production in total and that derived from NH4⁺-N and NO3⁻-N, respectively, in µg N2O-N kg⁻¹ soil. In addition we calculated the production of N2O-N as parts per mil (‰) of the amount of applied N, i.e. emission rate (total N, NH4⁺-N, or NO3⁻-N).
Data analysis: evaluation of the nitrifier contribution to $N_2O$

Wrage et al. (2005) suggested that the targeted $N_2O$ production pathways could be distinguished by analyzing both $^{18}O$ incorporation from $^{18}O$ enriched $H_2O$ as well as $^{15}N$ incorporation from $^{15}N$ enriched $NH_4^+$ and $NO_3^-$ into $N_2O$. However, the occurrence of $O$ exchange complicates data interpretation in this original approach (Kool et al., 2007; Kool et al., 2009b; Kool et al., 2009a). Our additional treatment with $^{18}O$ labeled $NO_3^-$ made it possible to partly quantify $O$ exchange. Here we propose a revised approach to the dual isotope method of Wrage et al. (2005) including the use of $^{18}O$ labeled $NO_3^-$ to evaluate the presence and potential significance of the nitrifier denitrification (ND) pathway to total $N_2O$ production.

To explore the potential presence and significance of nitrifier denitrification in our incubation experiment, we used the calculated actual $O$ incorporation from $H_2O$ into $N_2O$ ($AOI$) and the $O$ exchange during denitrification of $NO_3^-$ to $N_2O$ ($X_{ERR}$) derived from $^{18}O$ and $^{15}N$ labeling in addition to the above mentioned data derived from $^{15}N$ tracing ($N_2O_{(NH4)}$, $N_2O_{(NO3)}$, $N_2O_{FD}$ and $N_2O_{NCD-max}$). These calculations were described before in detail (Kool et al., 2009b; Kool et al., 2009a). A summary of the $AOI$ and $X_{ERR}$ derivation is provided in appendix 5-1 (eq A5.8 through eq A5.10) as well.

As we aimed to discern the potential extent of the nitrifier denitrification pathway, we confined our further data analysis to those soils where the combined nitrifier pathways (NN, ND, NCD) (total $N_2O_{(NH4)}$) accounted for at least 10% of total $N_2O$ production, or where absolute amounts of $NH_4^+$ derived $N_2O$ revealed a relevant nitrifier contribution ($N_2O-N>0.01\%$ of applied $NH_4^+\cdot N$).

Our evaluation is based on the $O$ incorporation from $H_2O$ into $N_2O$. We describe this approach and the calculations in detail in appendix 5-2. By calculating theoretical amounts of $O$ incorporation ($TOI$) and comparing these with the measured actual $O$ incorporation ($AOI$), we determined what the minimum and maximum contributions of the pathways could have been in order to agree with the observed $AOI$ (appendix 5-2). When the relative contribution to $N_2O$ production ($N_2O_p$) and the $O$ incorporation from $H_2O$ into $N_2O$ during production ($OI(N_2O_p)$) was known for each pathway $p$, we could calculate the $^{18}O$ incorporation from $^{18}O-H_2O$ into $N_2O$ that should be measured (Kool et al., 2009b):
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\[ \text{OI}(\text{N}_2\text{O}) = \sum N_2O_p \cdot \text{OI}(N_2O_p) \]
\[ = N_2O_{FD} \cdot \text{OI}(N_2O_{FD}) + N_2O_{NCD} \cdot \text{OI}(N_2O_{NCD}) \]
\[ + N_2O_{ND} \cdot \text{OI}(N_2O_{ND}) + N_2O_{NN} \cdot \text{OI}(N_2O_{NN}) \]  
(eq 5.3)

Following this notion, we determined what TOI would be expected under certain assumptions on the contributions of the pathways to N₂O and the occurrence of O exchange (appendix 5-2, eq A5.11, eq A5.13, eq A5.16 for TOI₁, TOI₂ and TOI₃). We then determined whether these assumptions on the pathway contributions could hold, or whether they were violated and should be rejected based on the measured AOI. First we assessed whether the \(N_2O_{(NH4)}\) must have been at least partially derived through nitrifier denitrification (ND), i.e. whether \(N_2O_{ND}>0\), by evaluating a TOI under the assumption that \(N_2O_{ND}\) is zero (TOI₁, eq A5.11 and eq A5.12). Next, we assessed whether ND might have accounted for all \(N_2O_{(NH4)}\) (TOI₂, eq A5.13, eq A5.14, eq A5.15). Concurrently, we considered the potential contributions of the pathways nitrifier nitrification (NN) and nitrification-coupled denitrification (NCD) to the \(N_2O_{(NH4)}\), and to total N₂O production in general. The pathways evaluated to have had a minimum contribution (i.e. \(N_2O_{p}>0\)) are further quantified (eq A5.17 through eq A5.20).

**Statistical analyses: data accuracy**

The uncertainty of the average values over the replicates was quantified with the standard error:

\[ s_e(\bar{x}) = \sqrt{\frac{s^2(x)}{n_x}} \]
with \(s^2(x)\) the variance of the individual measurements, and \(n\) the number of replicates.

Parameters \(N_2O_{(NH4)}\), \(N_2O_{(NO3)}\), and \(XERR\) are defined as ratios (appendix 5-1):

\[ N_2O_{(NH4)} = 100 \cdot \frac{^{15}N(N_2O_{(TR4)})}{^{15}N(N_2O_{(TR3)}) + ^{15}N(N_2O_{(TR4)})} \]  
(eq 5.4)

\[ N_2O_{(NO3)} = 100 \cdot \frac{^{15}N(N_2O_{(TR4)})}{^{15}N(N_2O_{(TR3)}) + ^{15}N(N_2O_{(TR4)})} \]  
(eq 5.5)
Both for the numerator and denominator of these ratios we have replicate measurements. The $N_2O(NH_4)$, $N_2O(NO_3)$, and $X_{ERR}$ were estimated by the ratios of the averages over the replicates. The variance of these estimated ratios are approximated by a first-order Taylor linearization (Kendall et al., 1977). For instance, if we denote $^{15}N(N_2O(TR4))$ by $\bar{x}$ and $^{15}N(N_2O(TR3))$ by $\bar{y}$, and the sum of $\bar{x}$ and $\bar{y}$ by $\bar{z}$, then the variance of $N_2O(NH_4)$ can be approximated by:

$$X_{ERR} = 100 - ERR$$

$$ERR = 100 \frac{^{18}O(N_2O(TR2))}{^{15}N(N_2O(TR3))} \frac{^{15}N(NO_3_{(TR3)})}{^{15}O(NO_3_{(TR2)})}$$  \hspace{1cm} (eq 5.6)

Similarly, the variance of $N_2O(NO_3)$ can be approximated by substituting $\bar{y}$ for $\bar{x}$ in the above equation. By experimental design, $\bar{x}$ and $\bar{y}$ are independent, however the sample means $\bar{x}$ and $\bar{z}$ are logically dependent and $\bar{y}$ and $\bar{z}$ likewise. The covariance of $\bar{x}$ and $\bar{z}$, or $\bar{y}$ and $\bar{z}$, with $\bar{z} = \bar{x} + \bar{y}$ and $\bar{x}$ and $\bar{y}$ independent, equals $v(\bar{x})$ or $v(\bar{y})$ respectively. For the approximation of the variance of $X_{ERR}$, we define $^{18}O(N_2O(TR3))$ as $\bar{z}$ and $^{15}N(N_2O(TR3))$ as $\bar{z}$. Here, $\bar{x}$ and $\bar{z}$ are independent so their covariance is zero.

For an estimate of these approximated variances the true variances $v(\bar{x})$ and $v(\bar{z})$, and the squared expectations $\mu^2(\bar{x})$ and $\mu^2(\bar{z})$ and the product $\mu(\bar{x})\mu(\bar{z})$, are replaced by their unbiased estimators:

$$\hat{v}(\bar{x}) = \frac{s^2(x)}{n(x)}, \hspace{0.5cm} \hat{v}(\bar{z}) = \frac{s^2(z)}{n(z)}, \hspace{0.5cm} \hat{\mu^2}(\bar{z}) = (\bar{z})^2 - \frac{s^2(z)}{n(z)}$$

and $$\hat{\mu}(\bar{x})\hat{\mu}(\bar{z}) = \bar{x} \cdot \bar{z} - \text{cov}(\bar{x}, \bar{z}).$$

**Statistical analyses: linear regression**

Relations between the soil parameters (including microbial parameters) and $N_2O$ emissions were evaluated by linear regression analysis in GenStat eleventh edition (VSN international Ltd.). Analyses were carried out for total $N_2O$ production, both the relative and absolute contributions of $NH_4^+$ and $NO_3^-$ derived $N_2O$, the potential maximum contribution of the NCD and minimum of
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the ND pathway. To avoid pseudo-replication the averages were taken as response values. For the absolute productions averages were log-transformed (natural logs), and the relative contributions were logit transformed. As we have only sparse data, we decided to fit simple linear models only, i.e. models with one predictor. Data from soils F3 and F4 were not included in the regression analyses because they exhibited very marginal total N$_2$O production which would be likely to bias the results.

Results

Basic soil characteristics (pH, organic C content and C:N ratio), data on microbial biomass N and C, and the microbial community compositions from the PLFA analyses are reported in Table 5.1. In general, the forest (F) soils had low pH, contained highest organic C contents and had higher C:N ratios than the

Table 5.2: Production of N$_2$O during incubation. Absolute production (µg N$_2$O-N kg$^{-1}$ soil) and relative production as % of applied N (emission rates) for total and NO$_3^-$ derived N$_2$O, and the relative contribution of NH$_4^+$-N and NO$_3^-$-N to total N$_2$O (%).

<table>
<thead>
<tr>
<th>Soil</th>
<th>[N$_2$O] $^a$ µg N kg$^{-1}$ soil</th>
<th>emission rate (%)</th>
<th>NO$_3^-$ derived N$_2$O</th>
<th>[N$_2$O(NO$_3$)] $^b$ µg N kg$^{-1}$</th>
<th>emission rate (%)</th>
<th>% of total N$_2$O</th>
<th>NH$_4^+$ derived N$_2$O</th>
<th>[N$_2$O(NH$_4$)] $^b$ µg N kg$^{-1}$</th>
<th>emission rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.7 (0.3)</td>
<td>0.02</td>
<td>98.02 (0.48)</td>
<td>1.7 (0.37)</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>21.6 (2.5)</td>
<td>0.22</td>
<td>99.95 (0.02)</td>
<td>21.6 (0.43)</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>0.5 (0.0)</td>
<td>0.00</td>
<td>96.04 (1.24)</td>
<td>0.4 (0.01)</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>0.3 (0.0)</td>
<td>0.00</td>
<td>96.15 (1.74)</td>
<td>0.3 (0.01)</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>26.2 (9.8)</td>
<td>0.26</td>
<td>10.16 (0.95)</td>
<td>2.7 (0.05)</td>
<td>98.84 (0.95)</td>
<td>23.5 (0.47)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>1031.0 (74.2)</td>
<td>10.31</td>
<td>97.44 (0.26)</td>
<td>1004.6 (20.09)</td>
<td>2.56 (0.26)</td>
<td>26.4 (0.53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>46.2 (13.7)</td>
<td>0.46</td>
<td>56.71 (8.50)</td>
<td>26.2 (0.52)</td>
<td>43.29 (8.82)</td>
<td>20.0 (0.40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>924.3 (122.9)</td>
<td>9.24</td>
<td>97.50 (0.97)</td>
<td>901.2 (18.02)</td>
<td>2.50 (0.98)</td>
<td>23.1 (0.46)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>239.1 (20.6)</td>
<td>2.39</td>
<td>88.43 (0.48)</td>
<td>211.4 (4.23)</td>
<td>11.57 (0.48)</td>
<td>27.7 (0.55)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>219.5 (27.3)</td>
<td>2.19</td>
<td>76.33 (0.28)</td>
<td>167.5 (3.35)</td>
<td>23.67 (0.28)</td>
<td>51.9 (1.04)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A3</td>
<td>95.4 (10.8)</td>
<td>0.95</td>
<td>70.14 (0.60)</td>
<td>66.9 (1.34)</td>
<td>29.86 (0.60)</td>
<td>28.5 (0.57)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>146.3 (14.3)</td>
<td>1.46</td>
<td>86.46 (0.19)</td>
<td>126.5 (2.53)</td>
<td>13.54 (0.19)</td>
<td>19.8 (0.40)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ values in brackets denote the standard error of the mean

$^b$ values in brackets denote the estimated standard error
grassland (G) and agricultural (A) soils. The organic C content was higher in the G soils than in the A soils, but their C:N ratios were comparable. Total microbial biomass N and C were lower in the agricultural (A) soils than in the forest (F) and grassland (G) soils. This was also the case for the PLFA biomass, both for total biomass and for the specified functional groups.

The data on N$_2$O production are listed in Table 5.2. Absolute production (µg N$_2$O-N kg$^{-1}$ soil) and relative production as % of applied N (emission rates) are given for total and NH$_4^+$ and NO$_3^-$ derived N$_2$O, as well as the relative contribution of NH$_4^+$-N and NO$_3^-$-N to total N$_2$O (%). These data were partly presented before (Kool et al., 2009b; Kool et al., 2009a). The estimated standard error of N$_2$O(NH$_4^+$) and N$_2$O(NO$_3^-$) was small for most soils, except for F3 and F4 which showed very little N$_2$O production. The contribution of NO$_3^-$ derived N$_2$O shows a wide range across soils, in both absolute terms and as % of total N$_2$O. The relative contribution of NH$_4^+$ derived N$_2$O as % of total N$_2$O varies considerably as well. However, in absolute amounts and emission rate (% of applied N) the NH$_4^+$-N contribution is notably similar across the G and A soils.

Table 5.3: Results of the TOI evaluation, including the AOI and $X_{ERR}$ that were used for the TOI calculations (appendix 5-1 and 5-2). The TOI are theoretical amounts of O incorporation from H$_2$O under specific assumptions on the pathway contributions to N$_2$O production and O exchange. The $X_{ERR}$ is the quantified O exchange during denitrification, the AOI is the actual O incorporation from $^{18}$O-H$_2$O into N$_2$O (Kool et al., 2009b).

<table>
<thead>
<tr>
<th>Soil</th>
<th>$X_{ERR}$</th>
<th>AOI</th>
<th>TOI$_1$</th>
<th>TOI$_2$</th>
<th>TOI$_3$</th>
<th>Implications TOI$_{1,2,3}$ for N$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>32.2 (12.33)</td>
<td>74.6 (1.2)</td>
<td>18.47</td>
<td>48.2</td>
<td>79.3</td>
<td>&gt;0 = $N_2O(NH_4)$ =0 &gt;0</td>
</tr>
<tr>
<td>G2</td>
<td>90.4 (0.73)</td>
<td>98.2 (0.4)</td>
<td>-</td>
<td>89.4</td>
<td>90.6</td>
<td>=0 = $N_2O(NH_4)$ =0 =0</td>
</tr>
<tr>
<td>G3</td>
<td>78.7 (5.01)</td>
<td>65.4 (14.0)</td>
<td>78.91</td>
<td>66.3</td>
<td>87.3</td>
<td>=0 &lt; $N_2O(NH_4)$ &gt;0 &gt;0</td>
</tr>
<tr>
<td>G4</td>
<td>82.2 (0.84)</td>
<td>89.0 (1.0)</td>
<td>-</td>
<td>81.4</td>
<td>82.7</td>
<td>=0 = $N_2O(NH_4)$ =0 =0</td>
</tr>
<tr>
<td>A1</td>
<td>95.6 (0.29)</td>
<td>95.9 (1.1)</td>
<td>95.81</td>
<td>90.3</td>
<td>96.1</td>
<td>&gt;0 = $N_2O(NH_4)$ =0 &gt;0</td>
</tr>
<tr>
<td>A2</td>
<td>96.8 (0.17)</td>
<td>102.5 (0.3)</td>
<td>94.64</td>
<td>85.7</td>
<td>97.5</td>
<td>&gt;0 = $N_2O(NH_4)$ =0 =0</td>
</tr>
<tr>
<td>A3</td>
<td>95.3 (0.22)</td>
<td>104.9 (0.4)</td>
<td>88.32</td>
<td>81.8</td>
<td>96.7</td>
<td>&gt;0 = $N_2O(NH_4)$ =0 =0</td>
</tr>
<tr>
<td>A4</td>
<td>87.3 (0.45)</td>
<td>95.4 (0.4)</td>
<td>88.37</td>
<td>82.2</td>
<td>88.9</td>
<td>&gt;0 = $N_2O(NH_4)$ =0 =0</td>
</tr>
</tbody>
</table>

$^a$ values in brackets denote the estimated standard error
$^b$ values in brackets denote the standard error of the mean
$^c$ only relevant when N$_2O(NH_4)$CD-max < N$_2O(NH_4)$
Nitrifier denitrification can be a source of N₂O from soil.

The [N₂O(NH₄)] was in the range of 20 to 30 µgN₂O-N kg⁻¹ soil for most G and A soils, with A2 as an upper outlier (51.9 µg N₂O-N kg⁻¹ soil). In the F soils on the contrary, the NH₄⁺-N contribution to N₂O was negligible.

Table 5.3 provides the calculated TOIs and the result of their assessment regarding the potential contributions of the nitrifier pathways to N₂O production. The (previously derived) XERR and AOI used for this evaluation are included. The further defined margins of the relative contributions of all pathways are listed in Table 5.4 as percentage of total production (Table 5.4a) and as percentage of NH₄⁺ derived N₂O (Table 5.4b). Figure 5.2 presents the identified minimum and maximum contribution of nitrifier denitrification (ND). The results identified that nitrifier denitrification had contributed to at least some of the N₂O production in most soils (Table 5.3, Table 5.4, Figure 5.2). In all soils except G3, ND may even have been responsible for up to 100% of the N₂O(NH₄) constituting up to 89.8% (G1) of the total N₂O production (Table 5.4, Figure 5.2). N₂O(NH₄) could also have been derived through nitrification-coupled denitrification (NCD), but the ¹⁵N tracing revealed that this contribution was constrained to less than all N₂O(NH₄) for most soils except G2 and G4 (N₂O(NCD-max, Table 5.4). For most other soils, part of

Table 5.4: The minimum and maximum potential contributions of the pathways to N₂O production (a) as % of total N₂O, and (b) as % of NH₄⁺ derived N₂O. The contribution of fertilizer denitrification (N₂O_FD) and the theoretical maximum contribution of nitrification-coupled denitrification (N₂O(NCD-max)) were derived previously (Kool et al., 2009b); the contributions of nitrifier denitrification (N₂O(ND-min) and N₂O(ND-max)) and nitrifier nitrification (N₂O(NN-min) and N₂O(NN-max)) were derived as described in appendix 5-2.
N₂O(NH₄) must have been produced through nitrifier denitrification (ND): the minimum contribution of ND was zero or negligible for G3 and A1, but comprised at least 73.8, 12.2, 27.9, and 4.2% of nitrifier-N₂O (N₂O(NH₄)) in soils G1, A2, A3 and A4 respectively (Table 5.4b, Figure 5.2).

For five of the eight soils, the nitrifier nitrification (NN) contribution was conclusively shown to be zero (G2, G4, A2, A3, and A4, Table 5.4a). There was no minimal contribution of NN confirmed for any soil except G3 (Table 5.3), and the maximum potential contribution was less than 10% of total N₂O for all soils (Table 5.4a). The highest N₂O_NN-max was found for G3, at 8.5% of total N₂O production (Table 5.4a). It must be noted that the data of this single soil (G3),
Nitrifier denitrification can be a source of N₂O from soil

which was also the only soil where $N_{2O_{ND-max}}$ was not equal to $N_{2O(NH4)}$ and $N_{2O_{ND-min}}$ could have been zero, displayed a relatively large variation in the primary data (Table 5.2).

For G2 and G4, the applicability of the TOI approach was limited because the TOI₁ is not relevant in those cases where $N_{2O_{NCD-max}}$ could have comprised the total $N_{2O(NH4)}$ ($N_{2O_{NCD-max}}$ is not constrained to less than $N_{2O(NH4)}$ by the $^{15}$N tracing). For these soils therefore both $N_{2O_{NCD}}$ and $N_{2O_{ND}}$ could range from zero as minimum up to the total $N_{2O(NH4)}$ as maximum.

Regression analysis showed that none of the microbial parameters was a significant predictor for any of the N₂O variables, with the single exception of $N_{2O_{NCD-max}}$ which seemed to be negatively related to actinomycete PLFA biomass (Table 5.5). Of the non-microbial parameters, pH was a significant predictor of the relative contributions of NO₃⁻-N and NH₄⁺-N, and of theoretical maximum nitrification-coupled denitrification and nitrifier denitrification contributions ($N_{2O_{NCD-max}}$ and $N_{2O_{ND-max}}$) to total N₂O production. The variables of nitrifier-induced production ($N_{2O_{(NH4)}}$, $N_{2O_{NCD-max}}$ and $N_{2O_{ND-max}}$) were all positively related to pH, the $N_{2O(NO3)}$ intrinsically decreased with pH (as it is inversely related to $N_{2O(NH4)}$) (Table 5.5). The organic C content demonstrated a negative effect on absolute total and NO₃⁻ derived N₂O (Table 5.5). Absolute NH₄⁺ derived N₂O showed a negative relation with C:N ratio. The relative $N_{2O(NO3)}$ and $N_{2O(NH4)}$ were respectively positively and negatively related to C:N ratio (Table 5.5).

Discussion

The results from our study provide the best evidence so far that nitrifier denitrification can indeed occur in soils, and can do so at substantial rates (Table 5.4, Figure 5.2). Eight of the twelve incubated soils showed a considerable contribution of nitrifier-induced (NH₄⁺ derived) N₂O production. We found that nitrifier denitrification must have contributed to N₂O production in at least five of these eight soils in order to explain the observed O incorporation from H₂O into the produced N₂O (Table 5.4, Figure 5.2). In all eight soils, nitrifier denitrification may even have been responsible for virtually all NH₄⁺ derived N₂O. In contrast, N₂O production as by-product from nitrification had hardly occurred, if at all (Table 5.4).
Table 5.5: Results of the linear regression analyses between dependent variables on N$_2$O production and predictor variables on soil properties and soil microbial parameters. Predictor variables are considered significant when $P < 0.05$; $ns =$ non-significant.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>P</th>
<th>$R^2_{adj}$</th>
<th>Total N$_2$O</th>
<th>NO$_3^-$ derived N$_2$O</th>
<th>NH$_4^+$ derived N$_2$O</th>
<th>NCDmax$^a$</th>
<th>NDmax$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>µg N kg$^{-1}$soil</td>
<td>% of total N$_2$O</td>
<td>µg N kg$^{-1}$soil</td>
<td>% of total N$_2$O</td>
<td>µg N kg$^{-1}$soil</td>
</tr>
<tr>
<td>pH</td>
<td>$ns$</td>
<td></td>
<td>0.005</td>
<td>0.66</td>
<td>$ns$</td>
<td>0.005</td>
<td>0.66</td>
</tr>
<tr>
<td>Corg</td>
<td></td>
<td></td>
<td>0.037</td>
<td>0.41</td>
<td>$ns$</td>
<td>0.028</td>
<td>0.45</td>
</tr>
<tr>
<td>C/N</td>
<td>$ns$</td>
<td></td>
<td>0.012</td>
<td>0.56</td>
<td>$ns$</td>
<td>0.012</td>
<td>0.56</td>
</tr>
<tr>
<td>Microbial Biomass N</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
</tr>
<tr>
<td>Microbial Biomass C</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
</tr>
<tr>
<td>Total PLFA Biomass</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
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<td>$ns$</td>
<td>$ns$</td>
</tr>
<tr>
<td>Bacterial Biomass</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
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<tr>
<td>Fungal Biomass</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
</tr>
<tr>
<td>Actinomycete Biomass</td>
<td>$ns$</td>
<td></td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
</tr>
<tr>
<td>VAM Biomass</td>
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<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
<td>$ns$</td>
</tr>
</tbody>
</table>

Model fit$^b$

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Corg</th>
<th>C/N</th>
<th>C/N</th>
<th>Actin Bm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>6.76 (1.00)</td>
<td>10.82 (2.13)</td>
<td>6.79 (1.09)</td>
<td>10.82 (2.13)</td>
</tr>
<tr>
<td>Parameter</td>
<td>-0.05 (0.02)</td>
<td>-1.35 (0.34)</td>
<td>-0.05 (0.02)</td>
<td>1.35 (0.34)</td>
</tr>
</tbody>
</table>

$^a$ only includes G and A soils

$^b$ for significant predictors only; model parameters (and standard errors (se)) for the ln-transformed data
Nitrifier denitrification can be a source of N$_2$O from soil

In most soils, the dominant pathway of N$_2$O production was fertilizer denitrification (FD) (Table 5.4). The high moisture level was very likely the major determinant for this. Relative to FD, nitrifier denitrification (ND) and nitrification-coupled denitrification (NCD) may likely be suppressed because the required first step of ammonium oxidation is limited by more anaerobic conditions. At low O$_2$ levels, ND in turn is thought to be favored relative to nitrifier nitrification (NN) (Wrage et al., 2001), which may explain why we observed very little N$_2$O production through the latter pathway (Table 5.4). However, although the high moisture levels were expected to be sub-optimal for NN relative to the other pathways, ammonium oxidation did occur. It therefore remains remarkable that N$_2$O production as by-product from nitrification (NN) appears to be completely absent.

Nitrifier-induced N$_2$O production, i.e. total NH$_4^+$ derived N$_2$O, was evident in all grassland (G) and arable (A) soils (Table 5.2). In contrast, all forest (F) soils exhibited very little N$_2$O derived from NH$_4^+$. Remarkably, across the G and A soils the absolute amounts of NH$_4^+$ derived N$_2$O production were very similar, whereas fertilizer denitrification varied considerably. This may again suggest that nitrification was limited under the experimental conditions, so that differences in the potential for nitrifier N$_2$O production pathways across soils are less expressed.

Next to the moisture conditions, the presence and significance of N$_2$O production pathways may likely be related with soil properties such as pH, C content and microbial community composition (Knowles, 1982; Bock et al., 1986; Haynes et al., 1986; Paul et al., 1996).

The pH was a significant predictor of the relative contributions to N$_2$O production. At higher pH, the relative proportion of N$_2$O derived from NH$_4^+$ increased at the cost of NO$_3^-$ derived N$_2$O. In general, low soil pH may have inhibited the nitrifier pathways altogether (NH$_4^+$-N derived N$_2$O) in the F soils, which limited the evaluation of the relation between soil pH and the different nitrifier-induced N$_2$O production pathways to the G and A soils. However, the potential (theoretical maximum) contribution of NCD and ND to N$_2$O were positively related with soil pH as well. As nitrite oxidation seems more sensitive to low pH than ammonia oxidation (Anthonisen et al., 1976) and accumulating NO$_2^-$ levels can become toxic to ammonia oxidizers, we speculate that lower pH
might favor nitrifier denitrification (ND) over nitrification-coupled denitrification (NCD). Based on culture studies it is indeed thought that in general nitrification is favored at pH of approximately 6.5 and higher (Bock et al., 1986; Haynes, 1986; Stephen et al., 1998), though nevertheless nitrification is demonstrated in a wide variety of acid soils (Rosswall, 1982; Haynes, 1986; De Boer et al., 2001). In pure cultures N. europaea remains the most studied AOB, although Nitrosospira spp. are more common in soil (Stephen et al., 1998; Kowalchuk et al., 2001; Smith et al., 2001). Different clusters of the latter were demonstrated to favor different pH conditions, ranging from 4.2 to 7 (Stephen et al., 1996; Stephen et al., 1998). Heterotrophic nitrification and/or the presence of micro-sites for autotrophic nitrification have been thought to explain the occurrence of nitrification in acid soils (De Boer et al., 1991; Paul et al., 1996), but De Boer et al. (1991) also suggested that aggregated autotrophic bacteria may actually dominate nitrification at low soil pH. Overall, pH is clearly a driving factor in N₂O production in total, and may likely affect the relative significance of the contributions of the different pathways.

Soil organic C content is also considered to be a determining factor in N₂O production (Firestone, 1982; Haynes et al., 1986; Weier et al., 1993; Sánchez-Martín et al., 2008). Especially production through denitrification would be enhanced at higher C content, by providing an energy source for this heterotrophic process. However, absolute total and NO₃ derived N₂O showed to be negatively related to organic C content in our study. This could be explained by the suggestion that increased C availability leads to more complete denitrification with N₂ rather than N₂O as the end-product (Weier et al., 1993; Mathieu et al., 2006; Miller et al., 2009). The relative contributions of mineral N pools to total N₂O emission, however, seemed to be significantly affected by C:N ratios by promoting the NO₃ derived N₂O at the cost of N₂O(NH₄) at higher ratios.

The microbial community analyses revealed considerable variation across the soils. Drying and sieving of the soil prior to incubation will have affected the microbial population, so data should be compared only in a semi-quantitative way. Although these microbial analyses are not directly indicative of the processes undertaken by the microbial community, comparative differences in microbial biomass N, C, and PLFA could be reflected in the N₂O production as studies have suggested that microbial community may influence its production.
Nitrifier denitrification can be a source of N\textsubscript{2}O from soil and the N\textsubscript{2}O:N\textsubscript{2} ratio (Holtan-Hartwig et al., 2000; Dembreville et al., 2006). However, except for a relation between actinomycete biomass and \(N\textsubscript{2}O_{\text{NCD-max}}\), neither total N\textsubscript{2}O production nor the relative contributions of the distinguished pathways were shown to be related to the microbial community composition (Table 5.5). Many actinomycetes are indeed able to denitrify (Shoun et al., 1998), but Miller et al. (2008; 2009) also found that there was no significant relation between the abundance of denitrifiers and N\textsubscript{2}O emissions in their soil incubation studies. The here observed positive effect of actinomycete PLFA biomass on the \(N\textsubscript{2}O_{\text{NCD-max}}\) might also be explained by their saprotrophic nature. Their ability to degrade more resistant organic substances (Paul et al., 1996) may increase C availability to other organisms in the soil. This may favor denitrification relative to nitrification, and thereby positively affect the relative contribution of NCD. However, we might then expect to see this effect on the contribution of FD as well, but both absolute and relative NO\textsubscript{3}- derived N\textsubscript{2}O did not show a significant relation with actinomycete biomass. Overall, differences in microbial community composition as distinguished by PLFA analyses are not necessarily reflected in N\textsubscript{2}O production pathways, as similar microbial functions are spread across different functional groups and vice versa diverse functions can be undertaken by the same group. We suggest that future research would mainly benefit from combining isotope tracing with molecular techniques involving the analyses of functional genes rather than community composition.

This study comprised quite a wide range of soil types and land uses, including temperate, continental and Mediterranean climate regions. Differences in climatic origin of the soil may be of interest in examining the pathways of N\textsubscript{2}O production. For example, Crenshaw et al. (2008) concluded that fungi may be the main producers of N\textsubscript{2}O in semi-arid soil (2008). In the semi-arid soil studied by Sánchez-Martín et al. (2008), nitrifier denitrification was thought to be the main source of N\textsubscript{2}O in contrast to a temperate soil where (nitrification-coupled) denitrification was dominant. Ma et al. (2007) found that in Arctic soil the role of denitrifiers was minor and suggest that nitrifier denitrification was in fact the dominant pathway of N\textsubscript{2}O production. Although (semi-) arid and Arctic regions may not be a major contributor to total atmospheric N\textsubscript{2}O inputs, extending further investigations to such regions could advance our understanding of the intricate complexity of N\textsubscript{2}O formation in (agro-) ecosystems.
Conclusions

This study aimed to assess whether N₂O production through nitrifier denitrification in soil may be conclusively proven. Evaluation of the data from our isotopic labeling experiment revealed that O isotopic enrichment of the produced N₂O could not be explained without assuming the presence of nitrifier denitrification (ND) in some of our soils. We thus conclude that ND can indeed take place and constitute an important contribution to total N₂O production in actual soils. Further research remains needed to study how different soil types and variable conditions like moisture and fertilizer treatments affect the (relative and absolute) N₂O production through the distinctive pathways.

Acknowledgements

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Appendix 5-1: Summary of the derivations of $N_2O_{(NH4)}$, $N_2O_{(NO3)}$, $N_2OF_D$, $N_2O_{NCD-max}$, AOI and $X_{ERR}$

These calculations and the reasoning behind them are explained in detail in Kool et al. (2009b; 2009a). In short, the proportions of total $N_2O$ derived from $NH_4^+$ and $NO_3^-$, $N_2O_{(NH4)}$ and $N_2O_{(NO3)}$ respectively, were calculated from the $^{15}N$-$N_2O$ enrichment data. The relative contribution of FD to $N_2O$ production ($N_2OF_D$) was then defined as $N_2O_{(NO3)}$. The $N_2O_{(NH4)}$ comprised the sum of the relative contribution of nitrification (NN; $N_2O_{NN}$), nitrifier denitrification (ND; $N_2O_{ND}$), and nitrification-coupled denitrification (NCD; $N_2O_{NCD}$) pathways together:

\[
N_2O_{(NH4)} = 100 \cdot \frac{^{15}N(N_2O_{(TR4)})}{^{15}N(N_2O_{(TR3)}) + ^{15}N(N_2O_{(TR4)})} \quad (eq \ A5.1)
\]

\[
N_2O_{(NO3)} = 100 \cdot \frac{^{15}N(N_2O_{(TR3)})}{^{15}N(N_2O_{(TR3)}) + ^{15}N(N_2O_{(TR4)})} \quad (eq \ A5.2)
\]

\[
N_2O_{(NO3)} = N_2OF_D \quad (eq \ A5.3)
\]

\[
N_2O_{(NH4)} = N_2O_{NN} + N_2O_{ND} + N_2O_{NCD} \quad (eq \ A5.4)
\]

Next, the maximum possible contribution of NCD, $N_2O_{NCD-max}$ was derived based on the $^{15}N$ enrichment of the $N_2O$ and $NO_3^-$ resulting from application of $^{15}N$ enriched $NH_4^+$ (TR4). Nitrate is assumed to be an obligatory intermediate for NCD, i.e. the use of nitrite by heterotrophic denitrifiers is assumed to be minimal (as $NO_3^-$ was abundant and is energetically more profitable to use). Therefore, where the $^{15}N$ enrichment in the total $N_2O$ did not exceed the $^{15}N$ enrichment of the $NO_3^-$ (from TR4), the $N_2O_{(NH4)}$ could have exclusively originated from NCD. The $N_2O_{NCD-max}$ then equaled the total $N_2O_{(NH4)}$. When $^{15}N$-$N_2O$ exceeded the $^{15}N$ enrichment in the $NO_3^-$ in TR4, the $N_2O_{NCD-max}$ comprised a fraction of the $N_2O_{(NH4)}$ that could be derived from the $^{15}N$ enrichment data (from TR4)(Kool et al., 2009b):

\[
If ^{15}N(N_2O_{(TR4)}) \leq ^{15}N(NO_3^-(TR4)) , then \ N_2O_{NCD-max} = N_2O_{(NH4)} \quad (eq \ A5.5)
\]

\[
If ^{15}N(N_2O_{(TR4)}) > ^{15}N(NO_3^-(TR4)) , then \ N_2O_{NCD-max} < N_2O_{(NH4)} \quad (eq \ A5.6)
\]

\[
N_2O_{NCD-max} = N_2O_{(NH4)} \left( \frac{^{15}N(NO_3^-(TR4))}{^{15}N(NO_3^-(TR4)) + ^{15}N(NH_4^+(TR4))} \right) \quad (eq \ A5.7)
\]
Chapter 5

From the application of $^{18}$O enriched H$_2$O (TR1), the actual O incorporation from H$_2$O into N$_2$O (AOI, %) was determined. It is calculated from the measured $^{18}$O enrichment of N$_2$O relative to the applied enrichment of the $^{18}$O-H$_2$O:

$$AOI = 100 \cdot \frac{^{18}O(N_2O_{(TR1)})}{^{18}O(H_2O_{(TR1)})}$$  \hspace{1cm} (eq A5.8)

The O exchange during denitrification, X$_{ERR}$, was quantified from the $^{18}$O:$^{15}$N enrichment ratio of the N$_2$O relative to that of the applied NO$_3^-$ (TR2 and TR3). Without O exchange, the retention of this enrichment ratio from NO$_3^-$ into N$_2$O (ERR) would be 100%. The exchange was thus quantified as the loss in this enrichment ratio, i.e. 100% minus the ERR:

$$X_{ERR} = 100 - ERR$$  \hspace{1cm} (eq A5.9)

$$ERR = 100 \cdot \frac{^{18}O(N_2O_{(TR2)})}{^{15}N(NO_3^-_{(TR2)})} \cdot \frac{^{15}N(NO_3^-_{(TR3)})}{^{18}O(N_2O_{(TR3)})}$$  \hspace{1cm} (eq A5.10)

Appendix 5-2: Evaluation of nitrifier denitrification

Identification of the presence of ND

With the use of the previously derived data from $^{18}$O and $^{15}$N tracing, we further evaluated the N$_2$O $^{18}$O enrichment data in order to assess the potential contribution of the nitrifier pathways to N$_2$O production. Basic assumptions on the O incorporation into N$_2$O through the different pathways were that (i) N$_2$O resulting from NN would not have any O incorporated from H$_2$O (only from O$_2$), (ii) the N$_2$O resulting from ND and NCD would obtain respectively 1/2nd and 2/3rd of the O from H$_2$O through reaction stoichiometry, and (iii) the OI for N$_2$O resulting from ND, NCD, and FD could be increased as an effect of O exchange, at the level of X$_{ERR}$ for all these pathways. For details on the derivation of the various OIs for the pathways under different assumptions regarding O exchange, we refer to Kool et al. (2009b).

We first identified whether ND must have contributed at least some part to total N$_2$O production. For this purpose we assumed its contribution to be zero, N$_2$O$_{ND} = 0$, and subsequently evaluate whether this assumption could hold or
Nitrifier denitrification can be a source of N₂O from soil

must be rejected in order to concur with the measured isotopic enrichments. We calculated the theoretical oxygen incorporation from H₂O (TOI) under these assumptions, TOI₁, by adopting the N₂O_NCD-max and ascribing the remaining N₂O (NH₄) to NN (N₂O_NN). By using N₂O_NCD-max and in addition assuming that NCD is affected by O exchange, the TOI was maximized. This TOI₁ thus amounted to:

\[
TOI₁ = N₂O(NO₃)_XERR + N₂O_{NCD-max} \cdot (2/3 + 2/3 \cdot XERR - 1/3 \cdot (XERR)^2) \quad (eq \ A5.11)
\]

Parameter settings for TOI₁:

\[\begin{align*}
N₂O_{FD} &= N₂O_{NO₃} \\
N₂O_{NCD} &= N₂O_{NCD-max} \\
N₂O_{ND} &= 0 \\
N₂O_{NN} &= N₂O_{NH₄} - N₂O_{NCD-max} \\
&\&
OI(N₂O_{FD}) &= XERR \\
OI(N₂O_{NCD}) &= 2/3 + 2/3 \cdot XERR - 1/3 \cdot (XERR)^2 \\
OI(N₂O_{ND}) &= n.a. \\
OI(N₂O_{NN}) &= 0
\end{align*}\]

When the AOI would be similar or lower than TOI₁, this would imply that no ND was needed to explain the ¹⁸O incorporation from H₂O into the produced N₂O (the AOI). However, when the AOI exceeded the TOI, part of the N₂O must have been produced through ND instead of NN to allow for ‘additional’ O incorporation to explain the AOI. This would identify a minimum value for N₂O_{ND}, N₂O_{ND-min}, and would simultaneously imply a maximum for the potential contribution of NN, N₂O_{NN-max}:

If AOI > TOI₁,

\[
then \quad N₂O_{ND-min} > 0 \& N₂O_{NN-max} < N₂O_{NH₄} - N₂O_{NCD-max} \quad (eq \ A5.12)
\]

Please note that TOI₁ would only be relevant when N₂O_{NCD-max} was constrained to less than N₂O_{NH₄} by the ¹⁵N tracing data. (We further quantified the N₂O_{ND-min} and N₂O_{NN-max} where relevant, as described below).

Next, we assessed the potential significance of the ND contribution. This was
done by conversely ascribing the \( N_2O_{(N\,H\,4)} \) completely to ND, i.e. \( N_2O_{ND} = N_2O_{(N\,H\,4)} \), to evaluate whether we had to identify a maximum to its contribution \( (N_2O_{ND-max}) \). For this TOI2 we minimized the theoretical O incorporation by assuming that O exchange did not take place under ND:

\[
\text{TOI}_2 = N_2O_{(NO\,3)} \cdot X\text{ERR} + N_2O_{(N\,H\,4)} \cdot 0.5 \tag{eq A5.13}
\]

Parameter settings for TOI2:

- \( N_2O\,FD \) = \( N_2O_{(NO\,3)} \)
- \( N_2O\,NCD = 0 \)
- \( N_2O\,ND \) = \( N_2O_{(N\,H\,4)} \)
- \( N_2O\,NN = 0 \)

\&

- \( OI(N_2O\,FD) = X\text{ERR} \)
- \( OI(N_2O\,NCD) = \text{n.a.} \)
- \( OI(N_2O\,ND) = 0.5 \)
- \( OI(N_2O\,NN) = \text{n.a.} \)

When the AOI would be smaller than TOI2, this would mean that not all of the \( N_2O_{(N\,H\,4)} \) could have originated from ND. It would simultaneously imply that part of the \( N_2O_{(N\,H\,4)} \) would need to be ascribed to NN (to obtain a lower TOI that better explains the AOI):

\[
\text{If } \text{AOI} < \text{TOI}_2, \text{ then } N_2O_{ND-max} < N_2O_{(N\,H\,4)} \& N_2O_{NN-min} > 0 \tag{eq A5.14}
\]

(Quantification of the minimum NN contribution, \( N_2O_{NN-min} \), follows below).

Conversely, when the AOI was not smaller than TOI2, all \( N_2O_{(N\,H\,4)} \) could indeed have been derived through ND, without proof for any minimum contribution to \( N_2O \) production through NN:

\[
\text{If } \text{AOI} \geq \text{TOI}_2, \text{ then } N_2O_{ND-max} = N_2O_{(N\,H\,4)} \& N_2O_{NN-min} = 0 \tag{eq A5.15}
\]
Nitrifier denitrification can be a source of N\(_2\)O from soil.

We also consider whether \(N_2O_{ND-max}\) should be limited (i.e. less than \(N_2O_{(NH4)}\)) because part of the \(N_2O_{(NH4)}\) would need to be assigned to NCD instead. Another TOI could be derived, for which all \(N_2O_{(NH4)}\) is again ascribed to ND but where O exchange is assumed to affect the \(OI(N_2O_{ND})\) as well. However, when O exchange is maximized (by being present during ND as well as during NO\(_2^\cdot\) oxidation to NO\(_3^-\)) the \(OI(N_2O_{ND})\) in fact equals the \(OI(N_2O_{NCD})\). In other words, assigning part of the \(N_2O_{(NH4)}\) to NCD would not further improve the TOI estimation of the AOI. Therefore, the conclusion of eq A5.15 remains.

However, it remained valuable to compare such a TOI that maximizes O exchange, TOI\(_3\), with our AOI as well:

\[
TOI_3 = N_2O_{(NO_3)} \cdot X_{ERR} + N_2O_{(NH4)} \cdot (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2) 
\]

(eq A5.16)

Parameter settings for TOI\(_3\):
\[
N_2O_{FD} = N_2O_{(NO_3)} \\
N_2O_{NCD} + N_2O_{ND} = N_2O_{(NH4)} \\
N_2O_{NN} = 0 \\
\& \\
OI(N_2O_{FD}) = X_{ERR} \\
OI(N_2O_{NCD}) = OI(N_2O_{ND}) = 2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2 \\
OI(N_2O_{NN}) = n.a.
\]

As this TOI\(_3\) is the ‘ultimate’ maximum TOI we could compute based on our data, it should not severely underestimate our AOI. When it closely estimates the AOI, we in fact recognize that the contribution of NN to N\(_2\)O production must have been negligible, i.e. \(N_2O_{NN-max}\) should be zero.

**Quantification of the \(N_2O_{NN-max}, N_2O_{ND-min},\) and \(N_2O_{NN-min}\)**

As described above (eq A5.12), when AOI > TOI\(_1\) we identified a minimum contribution of ND, i.e. \(N_2O_{ND-min} > 0\). Also, the contribution of NN must have been less than assumed under TOI\(_1\), i.e. \(N_2O_{NN-max} < N_2O_{(NH4)} - N_2O_{NCD-max}\). From the evaluation of TOI\(_2\) we may have confirmed a minimum contribution of NN to N\(_2\)O production, i.e. \(N_2O_{NN-min} > 0\) (eq A5.14). We now aimed to further quantify these \(N_2O_{ND-min}, N_2O_{NN-max}\) and \(N_2O_{NN-min}\).
First, evaluation of TOI 2 and TOI 3 could have determined that \(N_2O_{NN\text{-max}}\) was zero. In that case, all \(N_2O(NH_4)\) was derived through \(N_2O_{NCD}\) and \(N_2O_{ND}\). The minimum \(N_2O_{ND}\) is then derived using the \(N_2O_{NCD\text{-max}}\):

\[
\text{If } N_2O_{NN\text{-max}} = 0, \\
\text{then } N_2O_{ND\text{-min}} = N_2O(NH_4) - N_2O_{NCD\text{-max}}, \\
i.e. N_2O_{ND\text{-min}} = 100 - N_2O_{FD} - N_2O_{NCD\text{-max}} \tag{eq A5.17}
\]

When our data indicated that there may have been \(N_2O\) production through \(NN\), i.e. \(N_2O_{NN\text{-max}} > 0\), we quantified the \(N_2O_{NN\text{-max}}\) by determining what minimal contributions of the other pathways together would be required to explain the AOI. The \(N_2O_{NN}\) itself does not contribute to the AOI (\(OI(N_2O_{NN}) \equiv 0\)). We minimized the \(N_2O_{ND}\) and \(N_2O_{NCD}\) that would be needed to achieve the AOI by assuming that both are affected by O exchange (i.e. maximizing the \(OI(N_2O_{ND})\) and the \(OI(N_2O_{NCD})\)). As noted above, the OI for those pathways would be equal, so it does not matter (for the OI) whether the \(N_2O\) has been produced through ND or NCD. The combined contribution of ND and NCD to \(N_2O\) production is defined as \(N_2O_{(NCD\text{+ND})\text{-min}}\), and their OI denoted as \(OI(N_2O_{NCD\text{+ND}})\). We then calculated the \(N_2O_{NN\text{-max}}\) as follows:

\[
N_2O_{FD} + N_2O_{(NCD\text{+ND})\text{-min}} + N_2O_{NN\text{-max}} = 100 \\
AOI = N_2O_{FD} \cdot X_{ERR} + N_2O_{(NCD\text{+ND})\text{-min}} \cdot OI(N_2O_{NCD\text{+ND}}), \ i.e. \\
N_2O_{(NCD\text{+ND})\text{-min}} \cdot OI(N_2O_{NCD\text{+ND}}) = AOI - N_2O_{FD} \cdot X_{ERR}, \ i.e. \\
N_2O_{(NCD\text{+ND})\text{-min}} = (AOI - N_2O_{FD} \cdot X_{ERR}) / OI(N_2O_{NCD\text{+ND}}), \ where \\
OI(N_2O_{NCD\text{+ND}}) = 2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2 \\
N_2O_{NN\text{-max}} = 100 - N_2O_{FD} - N_2O_{(NCD\text{+ND})\text{-min}} \tag{eq A5.18}
\]

The \(N_2O_{ND\text{-min}}\) would then amount to \(N_2O_{(NCD\text{+ND})\text{-min}}\) minus the \(N_2O_{NCD\text{-max}}\):

\[
N_2O_{ND\text{-min}} = N_2O_{(NCD\text{+ND})\text{-min}} - N_2O_{NCD\text{-max}} \tag{eq A5.19}
\]

From the evaluation of TOI 2 we might have concluded that NN had a minimum contribution to \(N_2O\), i.e. \(N_2O_{NN\text{-min}} > 0\), if the AOI<TOI 2 (eq A5.14). To quantify the \(N_2O_{NN\text{-min}}\), we evaluated a theoretical scenario where O incorporation
Nitrifier denitrification can be a source of N$_2$O from soil is minimized. The N$_2$O$_{NCD}$ was thus set to zero and ND was not subject to O exchange, similar to the approach for TOI2. However, as TOI2 indicates (eq A5.14), part of the N$_2$O$_{(NH4)}$ needs to be assigned to NN instead of ND:

\[ N_2O_{(NH4)} = N_2O_{ND-max} + N_2O_{NN-min} \]

\[ AOI = N_2O_{FD} \times X_{ERR} + N_2O_{ND-max} \times OI(N_2O_{ND}), \text{ i.e.} \]

\[ N_2O_{ND-max} \times OI(N_2O_{ND}) = AOI - N_2O_{FD} \times X_{ERR}, \text{ i.e.} \]

\[ N_2O_{ND-max} = \frac{AOI - N_2O_{FD} \times X_{ERR}}{OI(N_2O_{ND})}, \text{ where} \]

\[ OI(N_2O_{ND}) = 0.5 \]

\[ N_2O_{NN-min} = N_2O_{(NH4)} - N_2O_{ND-max} \]  

(eq A5.20)
Abstract As soils comprise the premier source of the greenhouse gas nitrous oxide (N₂O), it is essential to understand its key N₂O production pathways. The potential of nitrifier denitrification as production pathway of N₂O has been well established in pure culture studies, but proof of its occurrence in terrestrial ecosystems has remained elusive. Only recently empirical research has confirmed that nitrifier denitrification can produce N₂O in soil, but its relative significance was minor as experimental moisture conditions favored nitrate driven denitrification. Here we assess the relative importance of nitrifier denitrification under a range of moisture regimes, including conditions less optimal for denitrification. Using a novel multi-isotope tracing approach we show that nitrifier denitrification can be a major contributor to total N₂O emission from soil. The role of nitrifier denitrification can be equally significant as that of N₂O produced as by-product of ammonia oxidation. With respect to total denitrifying activity, nitrifier denitrification dominated N₂O production under conditions sub-optimal for heterotrophic denitrification. We conclude that nitrifier denitrification is distinct from conventional nitrification and denitrification and affected idiosyncratically by environmental conditions. Accordingly, nitrifier denitrification should be routinely addressed as one of the major sources of N₂O from soil.
Chapter 6

Introduction

Nitrous oxide has become the third most important anthropogenic greenhouse gas (IPCC, 2007), and is today’s single most important ozone-depleting emission (Ravishankara et al., 2009). When aiming to mitigate N\textsubscript{2}O emissions, accurate understanding of the biochemical processes responsible for N\textsubscript{2}O production is crucial (Baggs, 2008). Although a wide range of processes has the potential to produce N\textsubscript{2}O, its production in soil is generally primarily attributed to nitrification and denitrification. Semantics may confuse this apparently simple paradigm, since various nitrifiers are able to denitrify as well. This nitrifier denitrification (ND) by ammonia oxidizing bacteria (AOB) has long been acknowledged in pure cultures (Hooper, 1968; Ritchie et al., 1972), and it has been suggested that ND could be a universal trait in beta-proteobacterial ammonium oxidizers, which are thought to be the dominant ammonium oxidizing bacteria in soil (Shaw et al., 2006). As it is well established that nitrifying micro-organisms contribute significantly to N\textsubscript{2}O emission from soils (Bremner, 1997), and as soils are the major source of N\textsubscript{2}O to the atmosphere (IPCC, 2007), insight in the potential of ND in soils is of global environmental interest. An increasing number of studies suggests that ND may contribute significantly to N\textsubscript{2}O production in soil (Webster et al., 1996; McLain et al., 2005; Wrage et al., 2005; Venterea, 2007), but definite proof has remained elusive due to methodological constraints (Wrage et al., 2001; Wrage et al., 2005; Kool et al., 2007). Only recently a novel multi-isotope tracing approach was presented (Kool et al., 2010) that accounts for the potential exchange of oxygen (O) between H\textsubscript{2}O and intermediate compounds of N\textsubscript{2}O production (Kool et al., 2007; Kool et al., 2009b). This enabled further discrimination of nitrifier denitrification (ND) as an N\textsubscript{2}O production pathway that is distinct from conventional nitrification (NN) and denitrification (FD and NCD, denitrification of applied -fertilizer- NO\textsubscript{3} and nitrification-coupled denitrification, respectively) (Figure 6.1). It provided best proof to date in soil-based experiments that ND can indeed produce N\textsubscript{2}O in soil. However, the relative contribution of ND to total N\textsubscript{2}O production was minor in this set-up, as production was dominated by FD (Kool et al., 2010). This may be explained by experimental conditions, which at 80% water holding capacity (WHC) were optimal for denitrification. To study the significance of ND under conditions less
Nitrifier denitrification as a distinct and significant source of \( \text{N}_2\text{O} \) from soil

Figure 6.1. Depiction of the major pathways of \( \text{N}_2\text{O} \) formation. We distinguish \( \text{N}_2\text{O} \) production from nitrifiers (ammonia oxidizers) through nitrification (NN) and nitrifier denitrification (ND), and from denitrifiers through reduction of \( \text{NO}_3^- \) produced from nitrification, i.e. nitrification-coupled denitrification (NCD), and reduction of applied \( \text{NO}_3^- \), i.e. fertilizer denitrification (FD).

Methods

Replicate samples of a poor sandy soil (pH 5.4) were incubated in glass jars for 28h after application of 50 mg NH\(_4^+\)-N kg\(^{-1}\) and 50 mg NO\(_3^-\)-N kg\(^{-1}\) soil, with treatment-specific isotopically enriched compounds: \(^{18}\text{O}\) labeled H\(_2\text{O}\) or NO\(_3^-\), or \(^{15}\text{N}\) labeled NO\(_3^-\) or NH\(_4^+\) (TR1, TR2, TR3 and TR4 respectively). Three moisture treatments were imposed, i.e. 50, 70, and 90% WHC. Lids were kept closed (airtight) during the incubation period. Analyses on a random selection of gas samples confirmed that O\(_2\) concentrations in the headspace had not notably declined during the incubation.

At the end of the incubation, \( \text{N}_2\text{O} \) production, soil mineral N content, and their relevant O and N isotopic signatures were determined (Kool et al., 2009a). From the \(^{15}\text{N}\) enrichment data the relative contributions of NH\(_4^+\)-N and NO\(_3^-\)-N to total \( \text{N}_2\text{O} \) production were derived (Kool et al., 2009b). Analyses of the \(^{15}\text{N}-\text{NH}_4^+\)
in the $^{15}$N-NO$_3^-$ labeling treatment confirmed that the contribution of DNRA as potential N$_2$O producing pathway was negligible. Oxygen exchange during production of N$_2$O from NO$_3^-$ reduction ($X_{ERR}$) was determined by the ERR method (Kool et al., 2009a) (using the data from the $^{18}$O- and $^{15}$N-NO$_3^-$ labeling treatments) and taken into account with further data evaluation. The potential minimum and maximum contribution of the different pathways to total N$_2$O production was calculated following the combined O and N isotope tracing approach presented by Kool et al. (2010). A summary of the main calculations of this approach is provided as supplementary information. The main assumptions underlying the approach are that (i) N$_2$O produced as byproduct of ammonia oxidation (NN) obtains all O from O$_2$ (no O incorporation from H$_2$O), (ii) O incorporation from H$_2$O into N$_2$O from FD, ND and NCD is respectively zero, 1/2$^{nd}$ and 2/3$^{rd}$ through reaction stoichiometry, and can be increased as an effect of O exchange at the level of $X_{ERR}$ for all these pathways, and that (iii) nitrate (not only nitrite) is an obligatory intermediate for nitrification-coupled denitrification (NCD). Table 6.1 presents total N$_2$O production, relative contributions of NH$_4^+$-N ($N_2O(NH4)$) and NO$_3^-$-N ($N_2O(NO3)$) to total N$_2$O production and several intermediate parameters of the data evaluation.

All treatments were replicated five times, which provided for the standard errors of the means of isotope enrichment data. The variables $X_{ERR}$, $N_2O(NH4)$ and $N_2O(NO3)$ are defined as ratios of averages of the replicates, for which standard errors were approximated by a first-order Taylor linearization (Kool et al., 2010). As $X_{ERR}$ is a key parameter in the analyses to derive the relative pathway contributions, we carried out a sensitivity analysis of this parameter. A full data evaluation was additionally carried out using the $X_{ERR}$ plus or minus its standard error in the calculations. Oxygen exchange was set to zero when $X_{ERR}$ was calculated to be negative in the evaluation.

A summary of the data calculations is given in appendix 6. More specifics about the incubation set-up, analyses, and data calculations can be found in previous work (Kool et al., 2009b; Kool et al., 2009a; Kool et al., 2010).

**Results and Discussion**

Table 6.1 lists the total N$_2$O production over the incubation and intermediate
Nitrifier denitrification as a distinct and significant source of $\text{N}_2\text{O}$ from soil

parameters of the data calculations. The therewith calculated relative pathway contributions to $\text{N}_2\text{O}$ production are presented in Table 6.2 and Figure 6.2.

Our results show that ND can be the prime contributor to total $\text{N}_2\text{O}$ production from soil (Figure 6.2). Nitrifier denitrification contributed more to $\text{N}_2\text{O}$ production than total conventional denitrification of NO$_3^-$ (FD plus NCD) at both 50 and 70% WHC. In the nearly water saturated soil (90% WHC), $\text{N}_2\text{O}$ production was, as expected, dominated by conventional denitrification of NO$_3^-$. In all moisture treatments ND constituted a major proportion of the NH$_4^+$ derived $\text{N}_2\text{O}$, ranging from minima of 30-50% to maxima of 60-100%. $\text{N}_2\text{O}$ production as a by-product of ammonia oxidation, i.e. NN, comprised maximally 44-64% of NH$_4^+$ derived $\text{N}_2\text{O}$. Nitrifier denitrification should therefore be considered as an equally important pathway of $\text{N}_2\text{O}$ production as NN and conventional denitrification (FD plus NCD).

In our incubation experiments, soils were amended with both NH$_4^+$ and NO$_3^-$ to realize the required isotopic enrichment. As nitrification in general proceeds slower than (heterotrophic) denitrification, it would be expected that the relative contribution of FD in these incubations is larger than it would be in the field under non-nitrate fertilized conditions. Therefore in actual ecosystems where NO$_3^-$ may be relatively more scarce, the potential contribution of ND to total $\text{N}_2\text{O}$ is likely to be even more significant.

Although they are not known to produce $\text{N}_2\text{O}$, Archaea are suggested to have a potential significant role in the NH$_4^+$ oxidizing community in soils (Leininger et

Table 6.1: Total absolute production of $\text{N}_2\text{O}$ over the incubation period and intermediate parameters of the calculations of the relative pathway contributions. AOI= actual oxygen incorporation (from H$_2$O into $\text{N}_2$O); TOI= Theoretical oxygen incorporation.

<table>
<thead>
<tr>
<th>Moisture treatment</th>
<th>Total production $\mu g\text{N}_2\text{O}-\text{N kg}^{-1}\text{soil (se)}$</th>
<th>$\text{N}_2\text{O(NO}_3^-)$ % (se) $^a$</th>
<th>$\text{N}_2\text{O(NH}_4^+$) % (se) $^b$</th>
<th>AOI % (se) a</th>
<th>TOI1 %</th>
<th>TOI2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% WHC</td>
<td>0.78 (0.06)</td>
<td>20.0 (1.4)</td>
<td>80.0 (0.0)</td>
<td>80.0 (0.0)</td>
<td>4.0</td>
<td>40.0</td>
</tr>
<tr>
<td>70% WHC</td>
<td>0.93 (0.07)</td>
<td>16.1 (1.9)</td>
<td>83.9 (0.9)</td>
<td>83.9 (0.9)</td>
<td>9.4</td>
<td>46.3</td>
</tr>
<tr>
<td>90% WHC</td>
<td>16.74 (2.15)</td>
<td>92.1 (0.0)$^b$</td>
<td>7.9 (0.4)</td>
<td>7.9 (0.4)</td>
<td>57.8</td>
<td>61.2</td>
</tr>
</tbody>
</table>

$^a$ values between brackets denote the approximated standard error

$^b$ the approximated variance was negative and therefore set to zero
Potential occurrence of archaeal ammonia oxidation however does not impair our findings on the contribution of different nitrifier pathways to N₂O emissions. On the contrary, if Archaea are in fact responsible for a significant part of the ammonia oxidation this would relegate the role of AOB in that process. This would again imply that the ammonia-derived N₂O that is interpreted as by-product from ammonia oxidation by AOB may often be overrated.

The extent of oxygen exchange may severely affect the ¹⁸O signature of N₂O (Kool et al., 2009a), and is evidently an important parameter in analyses to distinguish the significance of the different pathways (Kool et al., 2010). A sensitivity analysis of this parameter confirmed the robustness and general
outcome of our results: when O exchange was varied from plus to minus its standard error the data evaluation still showed ND to be a major contributor to N₂O production in soil (Table 6.2).

Moisture conditions are a well-known driver of N₂O production (Webster et al., 1996). Our results indicate that nitrifier denitrification and ‘conventional’ denitrification may each respond differently to moisture conditions. The relative importance of FD was considerably less at reduced soil moisture content than at 90% WHC, while the relative contribution of ND as percentage of NH₄⁺ derived N₂O did not strongly differ between moisture conditions (Figure 6.2). In terms of absolute production, both ND and FD declined with moisture content, but ND much less so than FD: about 60% versus 99% reduction respectively (Table 6.3).

The increased importance of ND relative to FD at lower moisture content is notable, as from theory one might argue that soil moisture content and related oxygen (O₂) availability control FD and ND alike. In both reduction processes the NO₃⁻ or NO₂⁻ acts as electron acceptor. Because the NO₂⁻ and NO reductases and genes encoding for these enzymes in AOB have been found to be similar to those in heterotrophic denitrifiers (Chain et al., 2003; Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007; Norton et al., 2008), enzyme synthesis and/or activity in the two pathways might be expected to respond similarly to O₂ availability. On

<table>
<thead>
<tr>
<th>Moisture treatment</th>
<th>% of total N₂O</th>
<th>% of NH₄⁺ derived N₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% WHC</td>
<td>FD 20.0</td>
<td>NCDmax 6.0</td>
</tr>
<tr>
<td></td>
<td>NCDmin 36.7</td>
<td>NDmax 56.9</td>
</tr>
<tr>
<td></td>
<td>NNNmin 23.1</td>
<td>NNmax 37.3</td>
</tr>
<tr>
<td></td>
<td>NCDmax 7.5</td>
<td>NNmax 45.8</td>
</tr>
<tr>
<td></td>
<td>NNNmin 11.1</td>
<td>NNmax 28.9</td>
</tr>
<tr>
<td></td>
<td>NNNmax 48.7</td>
<td></td>
</tr>
<tr>
<td>50% WHC</td>
<td>NDmin 56.9</td>
<td>NDmax 37.3</td>
</tr>
<tr>
<td></td>
<td>NNNmin 23.1</td>
<td>NNmax 37.3</td>
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<td>NNNmax 100.0</td>
<td>NDmin 46.7</td>
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<tr>
<td></td>
<td>NNNmax 71.1</td>
<td>NDmax 43.6</td>
</tr>
<tr>
<td>70% WHC</td>
<td>FD 16.1</td>
<td>NCDmax 6.1</td>
</tr>
<tr>
<td></td>
<td>NCDmin 24.2</td>
<td>NDmax 49.9</td>
</tr>
<tr>
<td></td>
<td>NNNmin 34.0</td>
<td>NNmax 53.6</td>
</tr>
<tr>
<td></td>
<td>NNNmax 7.3</td>
<td>NDmin 28.9</td>
</tr>
<tr>
<td></td>
<td>NNNmax 59.5</td>
<td>NDmax 40.5</td>
</tr>
<tr>
<td></td>
<td>NNNmax 63.8</td>
<td></td>
</tr>
<tr>
<td>70% WHC</td>
<td>NDmin 49.9</td>
<td>NDmax 53.6</td>
</tr>
<tr>
<td></td>
<td>NNNmin 34.0</td>
<td>NNmax 53.6</td>
</tr>
<tr>
<td></td>
<td>NNNmax 7.3</td>
<td>NDmin 28.9</td>
</tr>
<tr>
<td></td>
<td>NNNmax 59.5</td>
<td>NDmax 40.5</td>
</tr>
<tr>
<td></td>
<td>NNNmax 63.8</td>
<td></td>
</tr>
<tr>
<td>90% WHC</td>
<td>FD 92.1</td>
<td>NCDmax 0.6</td>
</tr>
<tr>
<td></td>
<td>NCDmin 3.9</td>
<td>NDmax 7.9</td>
</tr>
<tr>
<td></td>
<td>NNNmin 0.0</td>
<td>NNmax 0.0</td>
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<tr>
<td></td>
<td>NNNmax 7.1</td>
<td>NDmin 49.4</td>
</tr>
<tr>
<td></td>
<td>NNNmax 100.0</td>
<td>NDmax 43.6</td>
</tr>
<tr>
<td></td>
<td>NNNmax 43.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2: Relative contributions of the N₂O production pathways during the incubation period, including the results of the sensitivity analysis (SA range). For the sensitivity analyses all contributions were calculated with the XERR lowered or raised with its standard error (calculated FD and NCDmax are not affected by that). FD = (N₂O from fertilizer denitrification (from applied NO₃⁻)); NCD = nitrification-coupled denitrification; ND = nitrifier denitrification; NN = nitrifier nitrification, i.e. ammonium oxidation.
the other hand, profound (eco-)physiological differences between the microorganisms responsible for FD and ND, i.e. heterotrophic denitrifiers and autotrophic ammonia oxidizers, may conceivably induce different responses to environmental conditions. In FD, NO$_3^-$ reduction serves respiration, but generally more energy can be gained when O$_2$ is used as electron acceptor. Most denitrifiers favor O$_2$ over NO$_3^-$ even at quite low O$_2$ concentrations, thereby precluding FD activity. In ND the oxidation of ammonium is thought to provide the electron source for NO$_2^-$ reduction (Ritchie et al., 1972; Poth et al., 1985; Bock et al., 1995). The amount of energy available from this process is thought to be similar to the amount of energy available from aerobic ammonium oxidation to nitrite (Jetten et al., 1999; Wrage et al., 2001). When NO$_2^-$ is available from NH$_4^+$ oxidation, AOB could subsequently oxidize NH$_4^+$ with NO$_2^-$ (ND) just as well as with O$_2$(NN) to obtain a similar energy gain. Consequently, aerobic conditions would not need to inhibit ND. In a study by Ritchie and Nicholas (Ritchie et al., 1972) *Nitrosomonas europaea* indeed reduced NO$_2^-$ to N$_2$O under both aerobic and anaerobic conditions. Also Shaw et al. (2006) found all AOB strains tested capable of ND under aerobic conditions. On the other hand, both the production of N$_2$O and the N$_2$O:NO$_2^-$ ratio from nitrifiers in pure cultures have been found to decrease with increasing aerobicity (Goreau et al., 1980; Poth et al., 1985; Bock et al., 1995; Kester et al., 1997). The latter suggests that under aerobic conditions N$_2$O production as by-product of ammonia oxidation (NN) is more important than N$_2$O from nitrifier denitrification (ND). Altogether, aerobicity likely affects the occurrence of ND, but O$_2$ concentrations that repress heterotrophic denitrification do not necessarily constrain nitrifier denitrification to the same extent or through the same mechanisms. Despite similarities in their enzyme system, the biochemical

<table>
<thead>
<tr>
<th>Moisture treatment</th>
<th>FD</th>
<th>NCDmax</th>
<th>NDmin</th>
<th>NDmax</th>
<th>NNmin</th>
<th>NNmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% WHC</td>
<td>0.16</td>
<td>0.05</td>
<td>0.29</td>
<td>0.45</td>
<td>0.18</td>
<td>0.29</td>
</tr>
<tr>
<td>70% WHC</td>
<td>0.15</td>
<td>0.06</td>
<td>0.23</td>
<td>0.47</td>
<td>0.32</td>
<td>0.50</td>
</tr>
<tr>
<td>90% WHC</td>
<td>15.43</td>
<td>0.09</td>
<td>0.65</td>
<td>1.32</td>
<td>0.00</td>
<td>0.57</td>
</tr>
</tbody>
</table>
processes of ND and FD are apparently not regulated alike.

An alternative explanation for the occurrence of ND is a response to NO$_2^-$ toxicity (Poth et al., 1985; Stein et al., 1998; Beaumont et al., 2004b; Shaw et al., 2006). A decrease in N$_2$O production by *N. europaea* when co-cultured with a nitrite oxidizer (*Nitrobacter winogradskyi*) was ascribed to lower NO$_2^-$ concentration (Kester et al., 1997). Further, the ammonium monoxygenase enzyme has been shown to be inhibited by nitrite under both aerobic and anaerobic conditions (Stein et al., 1998), and NirK has been shown to be expressed aerobically by *N. europaea* in response to increasing nitrite concentrations (Beaumont et al., 2004b). However, it could be argued that if ND serves to reduce NO$_2^-$ toxicity it should be positively related to NO$_2^-$ production from NH$_4^+$ oxidation, which conflicts with the observation that under well-aerated conditions NO$_2^-$ reduction by AOB is limited or absent while NH$_4^+$ oxidation continues to produce NO$_2^-$ (Bock et al., 1995). On the other hand expression of the genes for denitrifying enzymes (*nirK*, *norB* and *nsc*) in AOB has been found to be inhibited by NH$_4^+$ (Schmidt, 2009). Certain levels of NH$_4^+$ might thus inhibit ND, until ongoing NH$_4^+$ oxidation leads to increased NO$_2^-$ and/or decreased NH$_4^+$ concentrations that respectively exceed and/or drop below a threshold level of NO$_2^-$ toxicity and ND inhibition. Clearly, our study was not set-up to test hypotheses on NO$_2^-$ toxicity or NH$_4^+$ inhibition as driving factors for ND. Nevertheless, the evident potential importance of this pathway should instigate future studies to further unravel the dynamics and driving factors of ND.

**Conclusion**

In summary, we found that the ND pathway is fundamentally distinct from other N$_2$O production pathways and responds idiosyncratically to environmental conditions. Our results show that ND may not only occur, but can indeed comprise a significant contribution to total N$_2$O production in soils, especially under conditions that are suboptimal for heterotrophic NO$_3^-$ denitrification. The contribution of ammonia oxidation by AOB as N$_2$O source can be severely overrated when nitrifier denitrification is neglected. This strongly argues for nitrifier denitrification to be routinely considered as separate potential premier N$_2$O production pathway in biogeochemistry.
Chapter 6

Acknowledgements

The authors would like to thank C. Bufe for her contribution to the lab work, and Dr. D. Brus for his assistance with the statistics. This research was financed by the NitroEurope IP, funded by the European Commission under the 6th framework program. J.W.v.G. is supported by a personal VIDI grant from the Netherlands Organization of Scientific Research/Earth and Life Sciences (NWO-ALW).
Appendix 6: Summary of data calculations

The actual O incorporation from H₂O into N₂O (AOI) is calculated from the ¹⁸O enrichment of the N₂O and H₂O in treatment TR1, ¹⁸O(N₂O(TR1)) and ¹⁸O(H₂O(TR1)) respectively:

\[
AOI = 100 \cdot \frac{¹⁸O(N₂O(TR1))}{¹⁸O(H₂O(TR1))}
\]

(eq A6.1)

The oxygen exchange between H₂O and intermediates of the N₂O production pathways during reduction of NO₃⁻ to N₂O, XERR, is calculated from the ¹⁸O and ¹⁵N enrichment of the N₂O in treatment TR2 and TR3 respectively, ¹⁸O(N₂O(TR2)) and ¹⁵N(N₂O(TR3)), and the imposed ¹⁸O and ¹⁵N enrichment of NO₃⁻ in those treatments, ¹⁸O(NO₃−(TR2)) and ¹⁵N(NO₃−(TR3)):

\[
X_{ERR} = 100 \cdot \left(1 - \frac{¹⁸O(N₂O(TR2))}{¹⁸O(NO₃−(TR2))} \right) \frac{¹⁵N(N₂O(TR3))}{¹⁵N(NO₃−(TR3))}
\]

(eq A6.2)

The proportions of total N₂O derived from NH₄⁺ and NO₃⁻, the \(N₂O(NH₄)\) and \(N₂O(NO₃)\), are calculated from the ¹⁵N enrichment in treatment TR3 and TR4, ¹⁵N(N₂O(TR3)) and ¹⁵N(N₂O(TR4)) respectively:

\[
N₂O(NH₄) = 100 \cdot \frac{¹⁵N(N₂O(TR4))}{¹⁵N(N₂O(TR3)) + ¹⁵N(N₂O(TR4))}
\]

(eq A6.3)

\[
N₂O(NO₃) = 100 \cdot \frac{¹⁵N(N₂O(TR3))}{¹⁵N(N₂O(TR3)) + ¹⁵N(N₂O(TR4))}
\]

(eq A6.4)

The relative contribution of FD to total N₂O, \(N₂O_{FD}\), is defined as \(N₂O_{NO₃}\):

\[
FD = N₂O_{NO₃} \quad (FD = N₂O_{FD} \text{ in Kool et al., 2010})
\]

(eq A6.1)

The maximum proportion of N₂O that could have been derived from NCD, NCDmax, is calculated from the ¹⁵N enrichment of the N₂O and NO₃⁻ resulting from treatment TR4, ¹⁵N(N₂O(TR4)) and ¹⁵N(NO₃−(TR4)):

If \(¹⁵N(N₂O(TR4)) \leq ¹⁵N(NO₃−(TR4))\), then NCDmax = \(N₂O_{NH₄}\)

If \(¹⁵N(N₂O(TR4)) > ¹⁵N(NO₃−(TR4))\), then NCDmax < \(N₂O_{NH₄}\), &

\[
NCD\ max = N₂O_{NH₄} \cdot \frac{¹⁵N(NO₃−(TR4))}{¹⁵N(NO₃−(TR4)) + ¹⁵N(NH₄−(TR4))}
\]

(eq A6.6)
The N$_2$O derived from NH$_4^+$ comprises the N$_2$O that is produced through NN, ND and NCD. The N$_2$O$_{(NH_4)}$ is the sum of either (A) the maximum contribution of NN (NN$_{max}$), the minimum of ND (ND$_{min}$), and the maximum of NCD (NCD$_{max}$), or (B) the minimum contribution of NN (NN$_{min}$), the maximum of ND (ND$_{max}$), and the minimum of NCD (NCD$_{min}$). A Theoretical Oxygen Incorporation from H$_2$O into N$_2$O (TOI) is calculated under (A) that maximizes the O incorporation (assuming overall presence of O exchange) while minimizing the contribution of ND (TOI$_1$). Under (B), the TOI$_2$ is calculated which maximizes the contribution of ND and encounters the minimum O incorporation, i.e. through reaction stoichiometry and O exchange during FD only. As follows:

\[
N_2O_{(NH_4)} = NN + ND + NCD \quad (eq \, A6.8)
\]

(i.e. \(N_2O_{(NH_4)} = N_2O_{NN} + N_2O_{ND} + N_2O_{NCD}\) in Kool et al., 2010)

(A): \(N_2O_{(NH_4)} = NN_{max} + ND_{min} + NCD_{max}\)  

(eq A6.9)

\[
TOI_1 = N_2O_{(NO_3)} \cdot X_{ERR} + NCDmax \cdot (2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2)
\]

If AOI \(\leq\) TOI$_1$,

then ND$_{min} = 0 \& (NCD+ND)_{min} = N_2O_{(NH_4)}$

If AOI > TOI$_1$,

then ND$_{min} > 0$

\& (NCD+ND)$_{min} = (AOI \cdot FD \cdot X_{ERR})/(2/3 + 2/3 \cdot X_{ERR} - 1/3 \cdot (X_{ERR})^2)$

ND$_{min} = (NCD+ND)_{min} - NCD_{max}$

NN$_{max} = N_2O_{(NH_4)} \cdot (NCD+ND)_{min}$

(B): \(N_2O_{(NH_4)} = NN_{min} + ND_{max}\) (NCD$_{min} = 0$)  

(eq A6.10)

\[
TOI_2 = N_2O_{(NO_3)} \cdot X_{ERR} + N_2O_{(NH_4)} \cdot 0.5
\]

If AOI \(\geq\) TOI$_2$,

then ND$_{max} = N_2O_{(NH_4)} \& NN_{min} = 0$

If AOI < TOI$_2$,

then ND$_{max} < N_2O_{(NH_4)} \& NN_{min} > 0$

ND$_{max} = (AOI \cdot FD \cdot X_{ERR}) \cdot 0.5$

NN$_{min} = N_2O_{(NH_4)} - ND_{max}$
Nitrifier denitrification as a distinct and significant source of N₂O from soil
Chapter 7

Oxygen exchange affects the oxygen isotopic signature of nitrate in soil

Abstract  Oxygen stable isotope analyses are commonly used in nitrate (NO$_3^-$) source determination studies. The source and fate of NO$_3^-$ are studied based on distinct O isotopic signatures from potential sources and production and consumption processes of nitrate. In particular, the $\delta^{18}$O differs between sources like fertilizer, atmospheric deposition, and microbial production (nitrification), and is affected by fractionation effects during its transformation processes as well. However, O exchange between O from NO$_3^-$ and H$_2$O is in those studies implicitly assumed not to affect the $\delta^{18}$O-NO$_3^-$. Here we show in a soil-based experiment that this assumption may not hold. In a short (24h) incubation experiment, soils were treated with $^{18}$O and $^{15}$N enriched NO$_3^-$. Production of NO$_3^-$ during the incubation would affect both the $^{18}$O and the $^{15}$N enrichment. Oxygen exchange could therefore be studied by examining the change in $^{18}$O relative to the $^{15}$N. In two out of the three soils, we found that the imposed $^{18}$O enrichment of the NO$_3^-$ declined relatively more than the imposed $^{15}$N-NO$_3^-$ enrichment. This implies that O exchange might indeed affect the O isotopic signature of NO$_3^-$, which has implications for NO$_3^-$ source determination studies. We suggest that O exchange should be considered as a defining factor of the O isotopic signature of NO$_3^-$ when studying its origin and fate in ecosystems.
Introduction

Increasing concentrations of nitrate (NO$_3^-$) constitute an important environmental concern: contamination of groundwater and eutrophication of surface waters are recognized undesirable consequences of increased use of nitrogen (N) fertilizer and animal manure, atmospheric deposition and discharge of sewage waste (Howarth et al., 1996; Galloway et al., 2003). However, the significance of NO$_3^-$ input from different sources is often not known quantitatively. Evidently, identifying the sources and evaluating the progress of the production and consumption of NO$_3^-$ in ecosystems is of environmental interest.

Analyses of the $\delta^{15}$N and $\delta^{18}$O signatures of NO$_3^-$ are commonly used to evaluate its sources and fate in ecosystems including groundwater, drainage water, and river catchments (e.g., Amberger et al., 1987; Durka et al., 1994; Burns et al., 2002; Wankel et al., 2006; Kendall et al., 2007; Burns et al., 2009). Different sources and processes are assumed to impose distinct isotopic signatures on the NO$_3^-$ in these systems (Figure 7.1): atmospheric deposition and synthetic fertilizers are relatively highly enriched in $^{18}$O; organic fertilizer (manure, slurry) has relatively high $\delta^{15}$N values; denitrification leaves the residual NO$_3^-$ pool relatively enriched in both isotopes, and; NO$_3^-$ produced from nitrification results in typically the lowest $\delta^{15}$N and $\delta^{18}$O signatures among the considered sources. The expected range of the $\delta^{18}$O of NO$_3^-$ from nitrification is derived from the relative contribution of O$_2$ and H$_2$O to the total O incorporated during the oxidation steps. O$_2$ contributes one atom during ammonia oxidation to hydroxylamine (NH$_2$OH), and H$_2$O contributes the other two O atoms during further oxidation to nitrite (NO$_2^-$) and NO$_3^-$ (Hollocher et al., 1981; Andersson et al., 1983; Hollocher, 1984; Kendall et al., 2007). The relatively low $\delta^{18}$O of soil H$_2$O (-25 to +4‰ SMOW, Amberger et al., 1987) explains the relatively low $\delta^{18}$O of NO$_3^-$ from nitrification compared to the other sources (Figure 7.1).

In multiple NO$_3^-$ source determination studies, the $\delta^{18}$O of the NO$_3^-$ is low compared with that of atmospheric deposition and fertilizer input, but closer to the range expected from biologically produced NO$_3^-$ through nitrification (-5 to -15‰ SMOW) (Figure 7.1). It is consequently reasoned that most NO$_3^-$ in these systems (e.g., groundwater, drainage water, or river catchments) is derived from microbial nitrification within the soil system (e.g., Spoelstra et al., 2001; Williard et...
Oxygen exchange affects the oxygen isotopic signature of nitrate in soil

Figure 7.1: Overview of the different O and N isotopic signatures in NO$_3^-$ from various sources (Kool et al. (2007), after Kendall (1998)). Along the y-axis the 18O of atmospheric O$_2$ and (soil) H$_2$O are indicated.

al., 2001; Burns et al., 2002; Mayer et al., 2002). However, as mentioned above, soil water has an even lower $\delta^{18}O$ than all above mentioned NO$_3^-$ sources, including nitrification-derived NO$_3^-$ which is still assumed to obtain part of its O from O$_2$ (at approximately +23.5‰ SMOW) (Figure 7.1). If exchange of O with H$_2$O would affect the NO$_3^-$ pool, it would thereby lower the $\delta^{18}$O-NO$_3^-$. Regardless of the original source of the NO$_3^-$, its ‘net’ $^{18}$O isotopic signature would partly be defined by the $\delta^{18}$O of H$_2$O depending on the extent of the O exchange. As a result, the contribution of nitrification NO$_3^-$ would then be overestimated at the expense of atmospheric deposition and fertilizer input.

Isotope fractionation during denitrification leaves the residual NO$_3^-$ relatively enriched in $^{15}$N and $^{18}$O, which is used to evaluate the progress of denitrification (e.g. Böttcher et al., 1990; Wassenaar, 1995; Aravena et al., 1998; Groffman et al., 2006; Panno et al., 2006). However, an effect of O exchange would lower the $\delta^{18}$O-NO$_3^-$ (as H$_2$O is depleted in $^{18}$O compared to NO$_3^-$), which would mask (part of) the enrichment effect of denitrification. As a result, the rate of denitrification might be underestimated.

In summary, multiple sources and processes are taken into account to evaluate the fate and origin of NO$_3^-$ based on the O isotopic signature. However,
consideration of O exchange with H2O as a defining factor of the δ18O-NO3- is lacking. If O exchange indeed occurs and affects the δ18O of the NO3- in ecosystems significantly, it would constitute a pitfall for NO3- source determination studies.

To identify an effect of O exchange on the O isotopic signature of the NO3- pool in an actual ecosystem, one would need to know (i) the exact sources of the NO3-, as well as (ii) the isotopic fractionation factors of its production and consumption (mainly nitrification and denitrification, respectively). Therefore, to investigate the potential occurrence and effect of O exchange on the 18O-NO3- signature, we carried out a soil incubation experiment in which we were in control of (i) the source of the NO3-, i.e. by application, and (ii) the (negligible) effect of isotopic fractionation, i.e. by the use of enriched compounds. By tracing the fate of 18O relative to 15N enrichment of the NO3- we studied O exchange as a potential defining factor of the O isotopic signature of NO3-.

Methods

Experimental set-up

Three soils were used in our experiment: two silt loam grassland soils from experimental field Easter Bush near Edinburgh, United Kingdom, that differed in fertilizer and grazing intensity (i.e. ‘moderate’ and ‘intensive’ management; Gm and Gi respectively), and one sandy, relatively poor, arable soil from experimental farm ‘Droevendaal’ near Wageningen, The Netherlands (A). Soils were dried at 40°C, sieved (2mm) and stored at 4°C until further use. Soil samples were incubated in glass jars (100 g soil per jar) and treated with 50 mg NO3-N kg⁻¹ soil. The applied NO3- was artificially enriched in either 18O at 2.0 atom% excess (TR1) or 15N at 30.0 atom% excess (TR2). Two times four replicate samples per treatment, allowing for two destructive sampling moments, were pre-incubated for seven days at 40% WHC and 16°C. Moisture content was raised to 80% WHC with the treatment application at the start of the incubation.

After treatment application, soil was destructively sampled for the start (t0) measurement as soon as possible (within 4h after application at the latest). At the end of the incubation 24 hours later, the t24 samples were taken. All samples were processed by KCl extraction (20 g moist soil with 50 ml 1M KCl)
immediately after sampling. The O isotopic enrichment (\(^{18}\text{O}\)) of the NO\(_{3}^{-}\) in TR1 was determined on the KCl extracts by the denitrifier method (Casciotti et al., 2002; Xue et al., 2010). For the TR2 samples, the \(^{15}\text{N}\) isotopic signature of the NH\(_{4}^{+}\) and NO\(_{3}^{-}\) were derived by microdiffusion technique (Kool et al., 2009b). Isotopic analyses were carried out at the UC Davis Stable Isotope Facility.

**Data evaluation**

The \(^{18}\text{O}\) and \(^{15}\text{N}\) enrichments of the NO\(_{3}^{-}\) at t24 were compared with those at t0. Changes in the enrichments over the incubation period were studied using t-tests, significant differences were identified at P<0.05 (\(\alpha = 0.05\)).

Next to the individual \(^{18}\text{O}\) and \(^{15}\text{N}\) enrichments, we also evaluated the \(^{18}\text{O}:^{15}\text{N}\) enrichment ratio of the NO\(_{3}^{-}\) (ERR\(_{\text{NO3}}\)) to investigate whether their enrichment relatively to each other changed during the incubation. The ratio at t24 (ERR\(_{\text{NO3}}\)\(_{t24}\)) was compared to t0 (ERR\(_{\text{NO3}}\)\(_{t0}\)) and together defined the enrichment ratio retention ERR\(_{\text{NO3}}\):

\[
\text{ERR}_{\text{NO3}}(\%) = 100 \cdot \frac{\text{ERR}_{\text{NO3}}(t_{24})}{\text{ERR}_{\text{NO3}}(t_{0})}
\]

(eq 7.1)

with

\[
\text{ERR}_{\text{NO3}} = \frac{^{18}\text{O}(\text{NO}_{3}^{-}(\text{TR1}))}{^{15}\text{N}(\text{NO}_{3}^{-}(\text{TR2}))}
\]

(eq 7.2)

where \(^{18}\text{O}(\text{NO}_{3}^{-}(\text{TR1}))\) and \(^{15}\text{N}(\text{NO}_{3}^{-}(\text{TR2}))\) are the \(^{18}\text{O}\) and \(^{15}\text{N}\) isotopic enrichments of the NO\(_{3}^{-}\) in TR1 and TR2 respectively, either both determined at t0 for ERR\(_{\text{NO3}}\)\(_{t0}\) or at t24 for ERR\(_{\text{NO3}}\)\(_{t24}\). As the ERR\(_{\text{NO3}}\) are ratios, we could not directly derive standard errors of the means from the replicates. Standard errors of the ERR\(_{\text{NO3}}\) were therefore approximated by a first-order Taylor linearization (Kendall et al., 1977; Kool et al., 2010).

With the use of enriched compounds, the fractionation effects during production and consumption of NO\(_{3}^{-}\) become negligible. Therefore, the ERR\(_{\text{NO3}}\) should not change over the course of the incubation, i.e. the ERR\(_{\text{NO3}}\) should be 100\% in the absence of O exchange. O exchange would cause a decrease in the ERR\(_{\text{NO3}}\) at t24 compared to t0, represented by a loss in the ERR\(_{\text{NO3}}\).

**Results and discussion**

Our results show that in all soils the \(^{18}\text{O}\) enrichment of NO\(_{3}^{-}\) decreased significantly over the course of the incubation time (24h), while the \(^{15}\text{N}\)
enrichment did not (Figure 7.2, Table 7.1). Moreover, the $ERR(NO_3)$ was approximately 80, 88, and 96% for the 3 different soils, confirming a loss in $^{18}O$ relative to the $^{15}N$ (Figure 7.3). Overall small standard errors support high accuracy of the isotopic enrichment data. One data point (one replicate of TR2(t0) ($^{15}N-NO_3$)) appeared to be an outlier and was therefore excluded from the data calculations in Figure 7.2 and 7.3. Results of all data analyses on the isotopic enrichments and the $ERR(NO_3)$ are presented in Table 7.1.

Our results suggest that O exchange indeed occurred and affected the O isotopic signature of NO$_3^-$ in at least two of the three soils. To our knowledge, no ecosystem-based study has experimentally identified an effect of O exchange on the O isotopic signature of NO$_3^-$. Dissimilatory nitrate reduction to ammonium (DNRA) or immobilization and re-mineralization of NO$_3^-$ to NH$_4^+$ for subsequent nitrification, such as the ‘immobilization-mineralization turnover concept’ suggested by Mengis et al. (2001), would also maintain the $^{15}N$ but not the $^{18}O$ enrichment. Our incubation period was kept deliberately short to exclude significant effects of these processes. Given the short incubation period however,
more severe effects of O exchange might be expected in real ecosystems with longer residence time of the NO₃⁻.

As we intend to study the potential effect of O exchange, we should consider whether this may have occurred within our samples after sampling as well. During sample storage or transport, O exchange will likely be minimal due to the high salt concentrations in the sample (KCl extracts) that reduce microbial activity. Also during the analytical procedure of the denitrifier method O exchange is assumed to be minimal: it is one of the selection criteria for the denitrifier strain used in this technique (Pseudomonas aureofaciens) (Casciotti et al., 2002). Moreover, this method has shown excellent repeatability and very good comparison with the silver nitrate method (Xue et al., 2010). Most importantly however, we could disregard such uncertainty in our approach as the enrichments are measured at the start as well as at the end of the incubation. Any O exchange after sampling that would affect the obtained ¹⁸O signature will thus affect both measurements, and thereby not interfere with our evaluation of the

Table 7.1: The measured ¹⁸O and ¹⁵N isotopic signatures of NO₃⁻ (in TR1 and TR2, respectively) and the ER(NO₃) and ERR(NO₃) derived from those data. T-tests evaluated differences between the isotopic signatures at t₀ and t₂₄. Data between brackets denote the standard error of the mean for the isotopic signatures, and the approximated standard error for the ER(NO₃). The data of soil A are provided in- and excluding one outlier of TR2 at t₀. Also including this data point, the ¹⁵N of the NO₃⁻ did not change significantly over the incubation period (whereas the ¹⁸O-NO₃⁻ did (TR1)).

<table>
<thead>
<tr>
<th>Soil</th>
<th>¹⁸O enrichment (TR1)</th>
<th>¹⁵N enrichment (TR2)</th>
<th>ER(NO₃)</th>
<th>ERR(NO₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t₀</td>
<td>t₂₄</td>
<td>t-test</td>
<td>t₀</td>
</tr>
<tr>
<td>Gm</td>
<td>0.829 (0.013)</td>
<td>0.629 (0.014)</td>
<td>p&lt;0.05</td>
<td>11.74 (0.16)</td>
</tr>
<tr>
<td>Gi</td>
<td>1.108 (0.017)</td>
<td>0.899 (0.012)</td>
<td>p&lt;0.05</td>
<td>14.50 (0.38)</td>
</tr>
<tr>
<td>A</td>
<td>1.718 (0.008)</td>
<td>1.639 (0.027)</td>
<td>p&lt;0.05</td>
<td>24.23 (0.26)</td>
</tr>
<tr>
<td>A²</td>
<td>1.718 (0.008)</td>
<td>1.639 (0.027)</td>
<td>p&lt;0.05</td>
<td>27.63 (3.40)</td>
</tr>
</tbody>
</table>

*Including an outlying data point in TR2(t₀)*
occurrence of $\text{O}$ exchange during the incubation period between those measurements.

If $\text{O}$ exchange is indeed a defining factor of the $\text{O}$ isotopic signature of $\text{NO}_3^-$, this would have significant implications for contemporary $\text{NO}_3^-$ source determination. The quantitative contribution of the factors defining the $\text{O}$ isotopic signature all entail some uncertainty: the isotopic signatures of the different sources as well as the extent of isotopic fractionation effects during production and consumption of $\text{NO}_3^-$ are only known within a certain range (Bedard-Haughn et al., 2003; Kendall et al., 2007). Results from experimental studies at natural abundance can therefore generally be explained within the available conceptual framework, and will not directly lead to suspicion of $\text{O}$ exchange without the use of artificially enriched compounds.

Observed discrepancies (both higher and lower) from the $\delta^{18}\text{O}$ of $\text{NO}_3^-$ which is expected based on reaction stoichiometry have been attributed to fractionation, microscale variability in $\delta^{18}\text{O}$ of $\text{O}_2$ and $\text{H}_2\text{O}$, and contributions of heterotrophic nitrifiers whose nitrifying mechanism may differ with respect to $\text{O}$ incorporation (Mayer et al., 2001; Burns et al., 2002; Kendall et al., 2007). Mengis et al. (2001)
Oxygen exchange affects the oxygen isotopic signature of nitrate in soil

observed $^{15}$N signatures of the soil NO$_3^-$ which corresponded to a major contribution of fertilizer as NO$_3^-$ source, but simultaneously found that the $^{18}$O-NO$_3^-$ signatures were significantly lower than that of the fertilizer. They suggest that the original $\delta^{18}$O of the fertilizer NO$_3^-$ in their agricultural soils has faded due to microbial immobilization followed by mineralization and nitrification of the NO$_3^-$. In studies on the origin and fate of ecosystem NO$_3^-$, O exchange is hardly considered as defining factor of the $\delta^{18}$O-NO$_3^-$. However, the potential presence and effect of O exchange should not come completely unexpected. First, O exchange may occur during nitrification, the production of NO$_3^-$. The final nitrification step of NO$_3^-$ production from NO$_2^-$ oxidation incorporates H$_2$O-O and is catalyzed by the enzyme nitrite oxidoreductase (Aleem et al., 1965; Bock et al., 1986). However, reduction of NO$_3^-$ to NO$_2^-$ is found to be brought about by this enzyme as well (Sundermeyer-Klinger et al., 1984; Wood, 1986). In other words, this process is reversible, which may provide the mechanistic explanation of the O exchange. If forward and reverse NO$_2^-$/NO$_3^-$ transformations take place concurrently, the involvement of H$_2$O-O in this step implies that all the O in NO$_3^-$ (and NO$_2^-$) can ultimately be replaced by O from H$_2$O. Furthermore, in the first steps of nitrification (ammonia oxidation to NO$_2^-$), microbially mediated exchange may occur between H$_2$O and nitrite (NO$_2^-$). Relatively early pure culture studies already associated O exchange with nitrifiers, both ammonia and nitrite oxidizers (Andersson et al., 1982; Kumar et al., 1983; DiSpirito et al., 1986). Recent pure culture studies on four different stains of ammonia oxidizing bacteria (AOB) reported an O exchange of 1 to 25% of the NO$_2^-$-O atoms (Casciotti et al. (2010)). As a result, microbially produced NO$_2^-$ and NO$_3^-$ will exhibit $\delta^{18}$O values that are closer to that of H$_2$O than expected based on reaction stoichiometry. Using a multi-box model to evaluate the $\delta^{18}$O-NO$_3^-$ in the ocean, Sigman et al. (2009) also suggested that as a result of O exchange less than one out of six of the O atoms in NO$_3^-$ originates from O$_2$, which is consistent with at least 50% O exchange. Casciotti et al. (2010) noted the discrepancy between their observed relatively low amounts of exchange (maximally 25%) compared to these model derived exchange rates. They suggest this may in part be explained by the fact that ammonia oxidizing Archaea (AOA) (may) significantly contribute to total nitrification in ocean waters, but may perform nitrification with alternative
reaction mechanisms, e.g. involving different enzymes. However, it has not been considered that O exchange during the consumption of NO$_3^-$ may have an effect on the NO$_3^-$ as well.

The O isotopic signature of nitrous oxide (N$_2$O), produced from $^{18}$O enriched NO$_3^-$ or from non-enriched NO$_3^-$ in the presence of $^{18}$O enriched H$_2$O, clearly showed to be affected by O exchange (Kool et al., 2009a). Pure culture studies on denitrifying bacteria have also presented O exchange affecting the substrate NO$_2^-$ (Garber et al., 1982; Shearer et al., 1988). In the stepwise reduction of NO$_2^-$ to N$_2$O the O exchange is suggested to be mainly associated with the NO$_2^-$ and NO reduction steps (Garber et al., 1982; Kool et al., 2007). Again, the reversibility of this step may well explain the potential occurrence of O exchange. Although studies on O exchange during denitrification have mainly focused on the steps of NO$_2^-$ and NO reduction, an effect on NO$_3^-$ may still be conceived. For studies on NO$_3^-$, it might appear speculative to suggest that O exchange during NO$_3^-$ reduction might affect the substrate’s O isotopic signature. However, for sulfate (SO$_4^{2-}$) it is generally acknowledged that the $\delta^{18}$O is affected not only by isotope fractionation, but by (varying degrees of) equilibration with H$_2$O (Fritz et al., 1989; Böttcher et al., 2001; Farquhar et al., 2008; Turchyn et al., 2010). Oxygen and sulfur isotope effects in SO$_4^{2-}$ during its bacterial reduction process were modeled by Brunner et al. (2005a; 2005b), incorporating the forward and reverse steps in the reduction. With this model observed patterns in isotope data from natural environments and laboratory studies could be better explained. Also for NO$_3^-$ reduction, such a model based on O isotope exchange effects could help to better explain oxygen isotope effects of residual NO$_3^-$ from denitrification.

**Conclusion**

Altogether, based on observations in pure culture studies and model comparisons, O exchange needs to be considered as a defining factor of the O isotopic signature of NO$_3^-$. Awareness about this effect on NO$_3^-$ source determination has been limited since experimental ecosystem-based studies which address this potential effect have remained lacking. Our experiment indicates that O exchange may indeed affect the O isotopic signature of NO$_3^-$ in actual soil ecosystems. Studies evaluating the source and fate of NO$_3^-$ in
Oxygen exchange affects the oxygen isotopic signature of nitrate in soil ecosystems based on its O isotopic signature may have been overestimating microbial nitrification as a source of NO₃⁻, and/or underestimating the progression of denitrification. We conclude that this experiment should instigate further and more elaborate ecosystem-based studies to identify the presence and effect of O exchange on the O isotopic signature of NO₃⁻. Ultimately, considering O exchange would improve the interpretation of O isotopic analyses in NO₃⁻ source determination studies.

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Chapter 8
General discussion
Recalling the main objectives

A particular deficiency in our understanding of \( \text{N}_2\text{O} \) production in soil concerns the pathway of ‘nitrifier denitrification’ (Figure 8.1). ‘Conventional’ autotrophic nitrification and heterotrophic denitrification have generally been considered as the prime sources of biochemical \( \text{N}_2\text{O} \) production in soils (Mosier et al., 1998; Pérez et al., 2001). However, soil-based studies increasingly suggest that nitrifier denitrification, i.e. nitrite reduction (denitrification) by autotrophic ammonia oxidizers (nitrifiers), might contribute significantly to \( \text{N}_2\text{O} \) from soil as well (Granli et al., 1994; Webster et al., 1996; Hütsch et al., 1999; Wrage et al., 2004a; McLain et al., 2005; Ma et al., 2007; Venterea, 2007; Sánchez-Martin et al., 2008). The potential for this pathway of \( \text{N}_2\text{O} \) production has been proven for various ammonia oxidizers in pure cultures (e.g. Hooper, 1968; Ritchie et al., 1972; Colliver et al., 2000; Shaw et al., 2006), but its actual occurrence and contribution to the total \( \text{N}_2\text{O} \) production in natural ecosystems has remained elusive.

To enable the distinction of \( \text{N}_2\text{O} \) produced by nitrifier denitrification, novel methodology was needed. Combined O and N stable isotope tracing has been suggested to offer such methodology. However, an approach based on current understanding of the origin of the O in \( \text{N}_2\text{O} \) from \( \text{O}_2 \) and \( \text{H}_2\text{O} \) (Wrage et al., 2005) does not adequately consider, let alone account for, the potential interference of O exchange. Consequently, my first objective was (i) to study, identify and quantify the process of O exchange between \( \text{H}_2\text{O} \) and intermediates of the \( \text{N}_2\text{O} \) production pathways, and its effect on the O isotopic signature of \( \text{N}_2\text{O} \) from soil. I subsequently (objective ii) aimed to develop and apply an advanced O and N isotope tracing approach that could distinguish nitrifier denitrification from ‘conventional’ nitrification and denitrification in soil-based studies. Anticipating that nitrifier denitrification would be successfully identified, my final objective (iii) was to evaluate the significance and idiosyncratic character of nitrifier denitrification as production pathway of \( \text{N}_2\text{O} \) in soil.

In this chapter I discuss the main findings of my research and their implications for our understanding of \( \text{N}_2\text{O} \) emissions from soils. The main assumptions underlying my approach are also discussed.
Oxygen exchange as defining factor of the O isotopic signature of N₂O

According to reaction stoichiometry, N₂O obtains its O from O₂ and H₂O in ratios distinct for the different pathways of N₂O production (Figure 8.1). It has generally been assumed that the O isotopic signature of the produced N₂O would subsequently reflect the isotopic signature of its substrate (e.g. NO₃⁻) and of O₂ and H₂O in the ratios designated by reaction stoichiometry (further only affected by fractionation) (Kim et al., 1990; Pérez, 2005; Wrage et al., 2005; Kendall et al., 2007). In other words, O exchange between H₂O and intermediate compounds of biochemical N₂O production has been assumed to be negligible. My research conclusively falsified this assumption, and I conclude that the presence of O exchange has been inappropriately neglected. My literature review (chapter 2) already suggested that assumptions on the negligibility of O exchange should be approached with extreme caution. In a wide range of soils I experimentally identified that O exchange strongly determined the O isotopic composition of N₂O (chapter 3). These findings necessitated the development of an advanced approach to discriminate the main pathways of N₂O production in soil. In this pursuit, I introduced the use of the ratio of the ¹⁸O:¹⁵N isotopic enrichment of N₂O relative to that of NO₃⁻. Tracing their enrichment ratio retention (ERR) allowed to not only identify, but also quantify the O exchange for the denitrification of NO₃⁻ to N₂O (chapters 3 and 4). Incorporating the effect of O exchange in advanced dual (O and N) isotopic tracing enabled to further distinguish nitrifier...
denitrification from ‘conventional’ nitrification and denitrification as pathways of N$_2$O production in soil. Additionally, in an exploratory study I demonstrated that next to N$_2$O, O exchange might affect the O isotopic composition of NO$_3^-$ in soil as well (chapter 7). These findings suggest that NO$_3^-$ source determination studies may also need to recognize O exchange as defining factor of the O isotopic signature to properly interpret the sources and cycling of NO$_3^-$ in ecosystems.

**Nitrifier denitrification as pathway of N$_2$O production in soil: experimentally identified**

Applying the advanced dual isotope approach, I studied the occurrence of nitrifier denitrification as N$_2$O production pathway in soil (chapters 5 and 6). Albeit with an uncertainty range, these studies now present compelling evidence that nitrifier denitrification indeed occurs in soils.

In my studies on a diversity of European soils (chapter 5), total N$_2$O was dominated by NO$_3^-$ driven denitrification under the moist experimental conditions. The identified minimum contribution of nitrifier denitrification to total N$_2$O remained low (over 60% for one soil, but less than 10% for the others), and its relative significance might therefore appear small. However, actual contributions may likely have been larger, as this minimum was defined under a rather extreme scenario, maximizing both O exchange and the contribution of nitrification-coupled denitrification to N$_2$O production. Moreover, assessment of the nitrifier-derived N$_2$O revealed that the contribution of nitrifier denitrification strongly dominated over the contribution of ‘conventional’ nitrification. Next, my experiments on a single soil at different moisture levels (chapter 6) showed that at moderate moisture content (50% and 70% WHC), nitrifier denitrification was responsible for more N$_2$O than ‘conventional’ denitrification. To conclude, these results show that nitrifier denitrification can constitute a significant contribution to soil-derived N$_2$O.

**Nitrifier denitrification as pathway of N$_2$O production in soil: environmental controls**

After recognizing nitrifier denitrification as distinct pathways of N$_2$O production
next to ‘conventional’ nitrification and denitrification, we ultimately aim to understand its environmental controls as well. Prime environmental regulators of N$_2$O production include soil moisture and O$_2$ conditions, pH and C availability (Firestone et al., 1989; Paul et al., 1996; Robertson et al., 2007). It is generally accepted that ‘conventional’ nitrification and denitrification are affected differently by these environmental controls. Although anticipated, for nitrifier denitrification its idiosyncratic response has never been experimentally established since proof of its mere presence in soil remained lacking.

In chapter 6 I demonstrate that nitrifier denitrification indeed responds idiosyncratically to soil moisture content. Nitrifier denitrification is thought to occur under marginally aerobic and/or short-term anaerobic conditions, as O$_2$ is needed for preceding ammonia oxidation and the denitrifying pathway would be similar to that of heterotrophic denitrifiers (Wrage et al., 2001). My results suggest that nitrifier denitrification is less repressed by increased aerobicity than ‘conventional’ denitrification. Under relatively aerobic conditions, N$_2$O production by nitrifier denitrification could be equally significant as N$_2$O evolved as by-product of ammonia oxidation.

In chapter 5 I evaluated soil pH and C content as possible predictors of the relative pathway contributions to N$_2$O production. Relative contributions of NH$_4^+$ and NO$_3^-$ derived N$_2$O showed to be related with both soil pH and soil C content. However, the overall dominance of NO$_3^-$-driven denitrification under the (relatively moist) experimental conditions complicated the assessment, and effects of these parameters may therefore be less pronounced. With the small contribution of NH$_4^+$ (i.e. total nitrifier contribution) to total N$_2$O, and the ability to only partially quantify the different nitrifier pathways, the contribution of nitrifier denitrification was not distinct enough to evaluate its individual relation with the diversity in pH and soil carbon.

It is however possible to speculate about the response of nitrifier denitrification to variations in soil pH and soil carbon content, in comparison with ‘conventional’ nitrification and denitrification. Bacteria denitrify preferably at higher pH, and nitrogen oxide reductases in the stepwise denitrification pathway are thought to be progressively inhibited with decreasing pH (Knowles, 1982). Based on identified similarities in the denitrifying enzymes of autotrophic nitrifiers and heterotrophic denitrifiers, nitrifier denitrification and ‘conventional’
denitrification may be hypothesized to be affected alike by pH. However, my results already showed that the observed enzymatic similarity does not necessarily generate similar responses to moisture conditions. Nitrification is observed in ecosystems across a wide range of pH, but based on pure culture studies on AOB nitrification is thought to prefer higher pH as well. Wrage et al. (2001) evaluated that while AOB may favor ammonia oxidation at a pH of 7, at pH 4 they might gain more energy from nitrifier denitrification.

Heterotrophic denitrification requires a, preferably readily available, C source and is thus directly affected by soil C content and quality. Autotrophic ammonia oxidizers are indirectly affected by SOC content and quality as it regulates NH$_4^+$ availability through mineralization and immobilization (Paul et al., 1996; Robertson et al., 2007). Heterotrophic denitrification and autotrophic nitrification will therefore respond differently to variation in SOC, but ammonia oxidation and nitrite reduction by AOB (i.e. nitrification and nitrifier denitrification) might be regulated alike by C availability. However, total soil microbial activity, and therefore O$_2$ consumption, is C dependent. Increased C availability could thereby reduce O$_2$ availability for AOB, improving conditions for nitrifier denitrification relative to ammonia oxidation.

**Main implications: Oxygen exchange between H$_2$O and intermediates of N$_2$O production**

Evidently, the presence of O exchange in soil has implications for source determination of nitrogen oxides based on their O isotopic signature.

If O exchange is not accounted for when distinguishing the pathways of N$_2$O production, we would overestimate the pathways that according to reaction stoichiometry produce N$_2$O with relatively more O from H$_2$O: nitrification-coupled denitrification and nitrifier denitrification. Nitrous oxide produced as by-product of ‘conventional’ nitrification and from denitrification of applied (fertilizer) NO$_3^-$ would be underestimated. The level of O exchange varied across soils and moisture conditions (chapters 3 and 6). This is not surprising, as already in pure cultures large variation in O exchange rates was observed across bacterial strains (Ye et al., 1991; Casciotti et al., 2002). In complex ecosystems such as soils with variability in microbial composition and activity across space and time, O
exchange will also be highly dynamic. This implies that the effect of O exchange will need to be quantified in concurrence with N₂O production in each experiment, i.e. it is an indispensable component of accurate O isotope tracing.

Implications of O exchange should also be considered for natural abundance studies. At natural abundance levels, reaction steps in the production of N₂O fractionate in favor of the lighter isotopes (¹⁶O and ¹⁴N) resulting in a relatively depleted product (N₂O) compared to the substrate. Likewise, reduction of N₂O to N₂ leaves the residual N₂O relatively enriched with the heavier isotopes (¹⁸O and ¹⁵N) due to isotopic fractionation. This effect on the N₂O isotopic signature is used to study the process of production and consumption N₂O (e.g. Schmidt et al., 2004; Wrage et al., 2004b; Pérez, 2005; Van Groenigen et al., 2005). If the N₂O production is affected by O exchange, the δ¹⁸O of the N₂O pool would (next to fractionation factors) be further defined by the O isotopic signature of the H₂O involved. This would lower the δ¹⁸O of the N₂O, as H₂O typically has a lower δ¹⁸O value than N₂O and its preceding compounds. As consumption leads to relative ¹⁸O enrichment of the N₂O pool, O exchange might lead to overestimation of production relative to consumption of N₂O.

In addition, I present that the implications of O exchange between H₂O and intermediates of N₂O production are not limited to N₂O source determination: O exchange could affect NO₃⁻ as well (chapter 7). In source determination of NO₃⁻, analyses of the δ¹⁸O signature are commonly used to discriminate between NO₃⁻ derived from e.g. atmospheric deposition, fertilizer, and microbial production (i.e. nitrification) (e.g. Amberger et al., 1987; Durka et al., 1994; Kendall et al., 2007). In these studies, O exchange is (implicitly) assumed to be negligible. The δ¹⁸O of NO₃⁻ is often relatively low compared to that of atmospheric and fertilizer input, and closer to what would be expected from nitrification. Again, the δ¹⁸O of soil H₂O is even lower, and O exchange would therefore decrease the δ¹⁸O of the total NO₃⁻ pool. As a result, respective studies in e.g. ground and drainage water and river catchments may have been overestimating the contribution of nitrification-derived NO₃⁻ at the expense of atmospheric and fertilizer input.

Main implications: Nitrifier denitrification as pathway of N₂O production in soil
Identifying nitrifier denitrification as a distinct and idiosyncratically controlled pathway of \( \text{N}_2\text{O} \) production in soil is a step forward in our process based understanding of \( \text{N}_2\text{O} \) production. Ultimately, such understanding is key to adequately predict and mitigate \( \text{N}_2\text{O} \) emissions to the atmosphere. The acknowledgement of nitrifier denitrification therefore imposes considerable implications for studies that aim to simulate and predict \( \text{N}_2\text{O} \) emissions from soil. Main challenges in such modeling studies result from the fact that \( \text{N}_2\text{O} \) from soil (i) can derive from multiple processes, (ii) is produced and consumed simultaneously, and (iii) is controlled by a large number of environmental variables (Li, 2000). Process-oriented models have been developed that include sub-models describing nitrification and denitrification and their response to environmental controls (DNDC (Li, 2000), DAYCENT (Del Grosso et al., 2005)). Clearly, a distinct role of nitrifier denitrification is not yet considered in these models. The lack of understanding of nitrifier denitrification, both its significance and its idiosyncratic response to environmental controls, may be a reason why current models struggle to adequately simulate and predict \( \text{N}_2\text{O} \) emissions. The distinct response of nitrifier denitrification to environmental parameters is not accounted for and models may consequently fall short in predicting total \( \text{N}_2\text{O} \) emissions. Ultimately, process-based models would need to consider a more diverse set of processes of \( \text{N}_2\text{O} \) production that respond individually to environmental parameters. Incorporating nitrifier denitrification may improve model performance, but without doubt will be a major challenge, specifically as long as the controlling factors remain poorly understood.

**Main assumptions**

The findings of my research contribute to and have implications for our understanding of \( \text{N}_2\text{O} \) production in soil, as discussed above. However, next to these implications, main assumptions underlying my approach should be discussed. Primarily, it is assumed that (i) \( \text{N}_2\text{O} \) derived as by-product from ammonia oxidation (nitrifier nitrification, NN) does not obtain any O from \( \text{H}_2\text{O} \); (ii) \( \text{NO}_3^- \) is the substrate and an obligatory intermediate for ‘fertilizer’ denitrification and nitrification-coupled denitrification (i.e. total ‘conventional’ denitrification); (iii) across the pathways where O exchange is considered to
occur, it takes place at the same rate as quantified for denitrification of NO$_3^-$ to N$_2$O; (iv) the addition of mineral N compounds (NH$_4^+$ and NO$_3^-$) needed to obtain the desired enrichment does not severely disrupt the system. Figure 8.2 illustrates these assumptions, which I discuss and assess in more detail below.

Re (i): Nitrous oxide derived from ammonia oxidation is thought to be a by-product of (incomplete) oxidation of hydroxylamine (Hooper et al., 1979; Arp et al., 2003). As the O in hydroxylamine has been shown to originate from O$_2$ and not from H$_2$O (Dua et al., 1979; Hollocher et al., 1981), O$_2$ is assumed to be the sole source of the O in N$_2$O resulting as by-product from ammonia oxidation (Figure 8.1). However, the exact mechanism of this step from hydroxylamine to N$_2$O is still unknown, implying some uncertainty about this assumption (chapter 2). Although available literature suggests the validity of this assumption, only a full description of the hydroxylamine-N$_2$O step would fully verify it.

Re (ii): In the $^{15}$N tracing, it is assumed that in nitrification-coupled denitrification NH$_4^+$ is completely nitrified to NO$_3^-$ which is subsequently reduced by denitrifiers (Figure 8.1). However, these denitrifiers might also directly take up and reduce NO$_2^-$ formed in the first step of nitrification. The contribution of nitrification-coupled denitrification would in that case be underestimated and identified as nitrifier denitrification instead. However, although heterotrophic denitrifiers can reduce NO$_2^-$ directly, NO$_3^-$ is energetically more profitable. Also, the intermediate NO$_2^-$ would need to be released by the nitrifiers and move through the soil to become available for those denitrifiers. In my studies, NO$_3^-$ was abundant (applied) and clearly readily denitrified under the experimental conditions (chapters 3-6). Altogether, I postulate that this justifies the assumption that NO$_3^-$ was intermediate for the large majority of N$_2$O produced through nitrification-coupled denitrification in the soil incubation studies performed.

Re (iii): The developed methodology quantifies O exchange for the reduction of NO$_3^-$ to N$_2$O. In the data evaluation, the potential of O exchange during other pathways is considered as well, for which the same exchange rate is assumed. Nevertheless, uncertainty remains whether it indeed occurs during the considered nitrifier pathways, and if so to what extent. These uncertainties are however taken into account through analyzing a range of scenarios regarding the possible occurrence of O exchange in the possible other pathways (chapter 4). The
most extreme scenario maximizes O exchange: a (minimum) contribution of
nitrifier denitrification is thereby only identified when the $^{18}$O signature of N$_2$O
could not be explained without it. As a result, this approach quantifies the
relative contributions to N$_2$O production in terms of ranges (i.e. with minima and
maxima) with respect to the nitrifier pathways (chapters 5, 6).

Re (iv): A major advantage of the application of enriched compounds with
stable isotope tracing approaches is that the effect of fractionation, i.e. the
preferential use of the lighter isotope and residual enrichment of the heavier
isotope, becomes negligible. However, eliminating the effect of fractionation
entails the addition of NH$_4^+$ and NO$_3^-$, which may disrupt the experimental
system. This approach would therefore not allow to quantitatively determine the
in-situ contribution of N$_2$O production pathways. In stead, it is designed to

Figure 8.2: Illustration of the main assumptions related to the advanced dual isotope
approach that is used to study the main pathways of N$_2$O production in soil.
provide an assessment of the significance of the different pathways relative to each other, and across soil types and environmental conditions.

In conclusion, I believe that the above argumentation justifies the assumptions underlying my approach, and that multi-isotope tracing provides a powerful tool to improve our understanding of N\textsubscript{2}O production pathways in soil. My study provided the first compelling evidence that, next to ‘conventional’ nitrification and denitrification, nitrifier denitrification is one of the main pathways of N\textsubscript{2}O production in soil.

**Alternative origins of N\textsubscript{2}O production**

Apart from conventional nitrification, denitrification and nitrifier denitrification, a wide variety of processes with the potential to produce N\textsubscript{2}O is acknowledged in literature. These include dissimilatory nitrate reduction to ammonia (DNRA) (Smith et al., 1981; Stevens et al., 1998), heterotrophic nitrification, co-oxidation of ammonia by methanotrophs (Yoshinari, 1985; Megraw et al., 1989; Mandernack et al., 2009), aerobic denitrification (Lloyd et al., 1987; Bell et al., 1991; Takaya et al., 2003), fungal denitrification (e.g. Bollag et al., 1972; Shoun et al., 1992; Hayatsu et al., 2008) and co-denitrification (e.g. Garber et al., 1982; Tanimoto et al., 1992b; Laughlin et al., 2002) (Figure 8.3). Even though their environmental significance remains topic of debate, the growing awareness of this variety of processes prompts a survey of our current understanding of these potential contributors to N\textsubscript{2}O production.

**Dissimilatory nitrate reduction to ammonia** forms a distinct pathway in the N cycle. The pathway of DNRA is not well understood but it has been shown that N\textsubscript{2}O can be produced during ammonification of NO\textsubscript{3}\textsuperscript{-} (Smith et al., 1981; Stevens et al., 1998). Some studies speculate that DNRA could account for a significant part of NO\textsubscript{3}\textsuperscript{-} reduction, also in soils (Caskey et al., 1979; Bonin et al., 1998; Stevens et al., 1998; Huygens et al., 2007; Wan et al., 2009). Disregarding N\textsubscript{2}O production by DNRA would overestimate the contribution of denitrification in isotope tracing studies, including the one in this thesis. Two types of DNRA are recognized: the first is coupled to fermentation, the second to sulphur oxidation (Burgin et al., 2007). Nitrate reduction through fermentative DNRA rather than denitrification is thought to be relatively favored in NO\textsubscript{3}\textsuperscript{-}-limited systems.
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(Nijburg et al., 1997; Tiedje 1988) and DNRA coupled to sulphur oxidation is found mainly in aquatic environments (Brettar et al., 1991; Brunet et al., 1996). In our studies, DNRA was therefore unlikely to be significant. This was verified by the insignificant $^{15}$N enrichment of the NH$_4^+$ after application of enriched NO$_3^-$ (chapters 3-6). However, the need remains to check for the absence or presence of DNRA in future N$_2$O source determination studies. In general, understanding the pathway and role of DNRA in nitrogen cycling remains a future challenge.

Apart from DNRA, most of the above mentioned additional sources of N$_2$O are distinguished not because they are different biochemical pathways, but because they involve different microbial groups capable of similar pathways: i.e. nitrification by heterotrophic bacteria, fungi and methanotrophs, and denitrification by (semi-)aerobic bacteria, fungi, and through co-denitrification. If the substrates and products of these processes are indeed similar, they cannot be individually distinguished with current isotope tracing approaches, including the ones outlined in this thesis. However, various organisms may likely act, react, and be controlled idiosyncratically by environmental factors even while carrying out similar pathways.

Besides autotrophic bacteria, many **heterotrophic bacteria and fungi** are capable of **nitrification** (Robertson et al., 2007; Laughlin et al., 2008). Oxidation of

![Figure 8.3: Depiction of the diversity of processes and organisms that (may) have the potential to produce N$_2$O, although their relative significance in soil remains controversial.](image-url)
NH₄⁺ by heterotrophic bacteria is thought to be enzymatically similar to that of AOB (Robertson et al., 2007; Hayatsu et al., 2008), and fungi appear to be capable of oxidizing both NH₄⁺ and organic N (Robertson et al., 2007; Laughlin et al., 2008). Because autotrophic nitrification is commonly thought to be increasingly inhibited with lower pH (Bock et al., 1986; Haynes, 1986; Stephen et al., 1998), nitrification in acid soils has often been thought to be mainly heterotrophic (De Boer et al., 1991; Paul et al., 1996; Papen et al., 1998). De Boer and Kowalchuk (2001) however stated in a review that although heterotrophs may contribute to some extent, autotrophic bacteria dominate the nitrifying community in soil. Also methanotrophs may co-oxidize NH₄⁺, resulting in concomitant release of N₂O (Yoshinari, 1985; Megraw et al., 1989; Mandernack et al., 2009). Some studies speculate that the contribution of methanotrophs to nitrification in soil and (freshwater) sediment may be considerable (Roy et al., 1994; Mandernack et al., 2000). Because of their different carbon requirements, the relative contribution of N₂O from autotrophic, methanotrophic, heterotrophic, and fungal nitrification may likely be affected by the supply and quality of SOC. Notably, Archaea have been suggested to have a significant role in the NH₄⁺ oxidizing community in soils as well (Leininger et al., 2006). Ammonia oxidizing Archaea have not been shown to produce N₂O, but the mechanisms and their contribution to nitrification remain to be further explained (Nicol et al., 2006).

For long, heterotrophic bacteria have been held primarily responsible for denitrification in soils. Next to recognition of denitrification by autotrophic ammonia oxidizers (i.e. nitrifier denitrification), fungal denitrification in soil has recently gained more attention (Shoun et al., 1992; Laughlin et al., 2002; Morozkina et al., 2007; Crenshaw et al., 2008; Hayatsu et al., 2008). As fungi often lack N₂O reductase, N₂O appears to be the main product of fungal denitrification (Shoun et al., 1992). This may have important ecological implications as changes in environmental conditions, e.g. nutrient and tillage management, can affect the fungal:bacterial ratio and as such their contribution to total denitrification (Lovell et al., 1995; Frey et al., 1999; Bittman et al., 2005; De Vries et al., 2006). Moreover, O₂ availability has been shown to control fungi and bacteria differently. Where bacterial denitrification generally requires anaerobic conditions and denitrifying enzymes are (from nitrite to nitrous oxide reductase) increasingly inhibited by O₂ (Knowles, 1982; Ferguson, 1994; Zumft, 1997), fungi are reported to be capable of...
denitrification under both aerobic and anaerobic conditions and may even need low levels of O2 (Tanimoto et al., 1992a; Zhou et al., 2001; Hayatsu et al., 2008). Conversely, several aerobic denitrifying bacteria have also been identified from diverse ecosystems including soils, suggesting that aerobic denitrification may not be a trivial source of N2O in soils (Lloyd et al., 1987; Bell et al., 1991; Patureau et al., 2000; Takaya et al., 2003). Another distinguished pathway of N2O production is co-denitrification, where NO3- or NO2- is combined with other nitrogenous compounds to produce N2O or N2. This process is most commonly recognized in denitrifying fungi (Shoun et al., 1992; Tanimoto et al., 1992b; Laughlin et al., 2002; Morozkina et al., 2007), but some studies have also identified bacteria (including actinomycetes) able to carry out co-denitrification (Garber et al., 1982; Kumon et al., 2002). Isotope (15N) labeling studies are suggested to enable the distinction between denitrification and co-denitrification. However, in ecosystems the evident complexity of N-transformations complicates the isolation and discrimination of those two processes from the wide spectrum of other N2O and/or N2 producing processes. Adding to the denitrifying community, several Archaea have also been shown to carry out dissimilatory reduction of NO3- via NO2-, NO and N2O to N2 (Werber et al., 1978; Volkl et al., 1993; Cabello et al., 2004). This pathway appears similar to the bacterial one (Zumft et al., 2007; Hayatsu et al., 2008), but genome sequencing has revealed differences in the genetic organization, structure and regulation of the genes (Philippot, 2002). Recently, genes encoding for potential homologues of nitrite reductases (NirK) have also been found in ammonia oxidizing Archaea from various environments (including soils) (Bartossek et al., 2010). Altogether the role of Archaea in denitrification and N2O production in natural ecosystems remains to be elucidated. Even less understood is the process of denitrification coupled to anaerobic methane oxidation (i.e. nitrate/nitrite-dependent anaerobic methane oxidation, N-DAMO). Although theoretically feasible, experimental proof and acknowledgement of this process was obtained only recently. Raghoebarsing et al. (2006) identified the first and up to now only microbial consortium that can oxidize methane anaerobically with denitrification serving as electron-acceptor. Understanding the process and ecological significance of N-DAMO, let alone quantifying a potential contribution to N2O production, is still far from feasible.

While recognizing that in soil ecosystems the role of many of the above
mentioned processes may likely be minor, the above synthesis challenges our conventional understanding of N₂O production. Altogether, the ‘conventional’ paradigm that addresses ‘nitrification and denitrification as main processes of N₂O production in soils’ has been attractively simple and convenient, but is no longer be legitimate. Foremost, my research strongly encourages to routinely consider nitrifier denitrification as one of the major sources of N₂O from soil.

**Understanding the origin of N₂O and its Oxygen: Future research directions**

My research elucidated several aspects of N₂O production in soil, and naturally also raises new questions that point to future research directions. Continued studies on the **process of O exchange** are needed to better understand and account for its control on the O isotopic signature of nitrogen oxides. The occurrence and extent of O exchange in pathways other than NO₃⁻ reduction to N₂O could not be quantified in my experiments, which imposed assumptions on the data evaluation. Literature contains several studies on O exchange by denitrifiers in pure cultures: future studies could include investigations of O exchange in pure cultures of nitrifiers, ammonia oxidizers as well as nitrite oxidizers. Based on these results, adjusted assumptions could be made on the occurrence of O exchange during nitrifier N₂O production in soil. This would improve the assessment of the relative pathway contributions to N₂O production with the advanced dual isotope approach. Insights in nitrifier-induced O exchange would also be valuable regarding the potential implications for NO₃⁻ source determination studies. Observations in pure culture studies could further unravel the extent and mechanism of O exchange and its effect on NO₃⁻. Ecosystem studies (on e.g. soils, sediments, aquatic systems) could further identify and potentially quantify O exchange with the use of ¹⁸O enriched compounds. Effective implications for NO₃⁻ source determination at natural abundance would need to be assessed subsequently.

Understanding the **pathways of N₂O production** is indispensable for the development of effective mitigation strategies for N₂O emissions to the atmosphere (Mosier et al., 1998; IPCC, 2007). The identification of nitrifier denitrification as distinct major pathway of N₂O production suggests that current
models should adjust their process-based modules and incorporate a more diverse set of N₂O production processes that respond individually to environmental parameters. However, the environmental controls of N₂O production through nitrifier denitrification remain to a large extent unclear. Moreover, with the growing awareness of the wide variety of potential N₂O production processes it is clear that it remains a major challenge to comprehend all pathways and organisms involved. Future research should therefore first be directed to further improve our process-based understanding of N₂O production processes.

Technologies to investigate pathways of N₂O production continue to advance rapidly, including the use of isotopomer ratios and molecular techniques. Analyzing the isotopomer composition is increasingly suggested as a promising tool in source determination of N₂O (Schmidt et al., 2004; Toyoda et al., 2005; Sutka et al., 2006; Ostrom et al., 2010). Such an approach evaluates the intramolecular site preference (SP) of the ¹⁵N in N₂O, at natural abundance. Where isotope tracing studies need to apply enriched compounds to discount the effect of isotopic fractionation, studying the isotopomer composition can be done without the need to disturb ecosystems with fertilizing compounds. However, ambiguity about the SP for different pathways and microbial communities currently limits the use of isotopomer ratios to assess the contributions of distinct pathways to N₂O production (Schmidt et al., 2004; Well et al., 2006; Ostrom et al., 2007; Ostrom et al., 2010). Future studies could attempt to further characterize distinct SP values and combine this tool with other stable isotope techniques. While recognizing the need for future investigation, recent studies have already suggested the potential of the δ¹⁸O/SP fingerprint of N₂O as a tool to identify the dominant production process of N₂O in soil (Well et al., 2008; Well et al., 2009).

Molecular techniques enable to determine the abundance of ammonia oxidizers (AOB and Archaea) and denitrifying bacteria in ecosystems by DNA and mRNA extraction. Successive PCR amplification by specific primers targets the functional genes encoding for specific enzymes that catalyze nitrification and denitrification (Kowalchuk et al., 2001; Philippot, 2002; Wallenstein et al., 2005; Leininger et al., 2006; Sharma et al., 2007). An extensive, solid set of primers is already available, but they do not amplify all variants of the targeted genes (Sharma et al., 2007). Moreover, genes encoding for the enzymes of the
denitrification pathway in ammonia oxidizing bacteria (i.e. for nitrifier
denitrification) (Casciotti et al., 2005; Cantera et al., 2007; Garbeva et al., 2007;
Norton et al., 2008) and ammonia oxidizing Archaea (Bartossek et al., 2010)
appear to be homologous to those in heterotrophic denitrifiers, but it is not clear
whether these would be amplified by the same primers. Altogether, such
techniques may not cover or differentiate certain distinct pathways. For this,
future research could invest in further extension of genetic databases to serve
improved primer design, by molecular studies on more diverse pathways and/or
organisms involved. Continuous improvement of molecular techniques offers
great potential to be combined with stable isotope approaches, to study the
relation between microbial community and N\textsubscript{2}O production pathways.

To conclude, despite their current constrains, stable isotope, isotopomer, and
molecular techniques are promising tools that deserve further development. Their
integrated use offers great potential to further unravel the significance and
environmental controls of the diverse pathways of N\textsubscript{2}O production at a process-
based level. A key challenge remains that such process-targeted methodology
often sets high specific requirements and/or may influence the system under
study. Such technologies currently do not suit the scale and complexity of field
studies. Conversely, ecosystem-based approaches allow little in-depth process-
based examination of the sources of N\textsubscript{2}O production. While ‘up-scaling’ to
increasingly realistic and inherently complex systems, from pure cultures to soil
lab-incubations to in-situ field work, we have to settle for a less comprehensive
understanding of N\textsubscript{2}O production. Ultimately, it is an interdisciplinary research
challenge to adopt a complementary approach in search for a joint process- and
ecosystem-based understanding on the origin of N\textsubscript{2}O.


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Nitrous oxide (N\textsubscript{2}O) is a greenhouse gas that contributes to global warming and to the depletion of stratospheric ozone. To reduce N\textsubscript{2}O emissions to the atmosphere it is important to understand how and where it is produced. Currently, many uncertainties remain about the different pathways of N\textsubscript{2}O production and their environmental controls. Globally, soils are the major source of N\textsubscript{2}O to the atmosphere. With my research I therefore aimed to improve our understanding on the production of N\textsubscript{2}O in soil ecosystems. Specifically, the main objective was to identify the presence and prospective contribution of ‘nitrifier denitrification’ as a distinct N\textsubscript{2}O production pathway. Nitrifier denitrification is the stepwise reduction of nitrite (NO\textsubscript{2}\textsuperscript{-}) to N\textsubscript{2}O and N\textsubscript{2} by ammonia oxidizing bacteria. In pure culture studies, the potential of these nitrifiers to produce N\textsubscript{2}O through this pathway had been well studied. However, experimental proof of the presence of nitrifier denitrification in actual soils remained inconclusive due to the lack of adequate methodology.

A novel dual isotope approach was suggested to enable the distinction of nitrifier denitrification from the conventionally considered two main pathways of N\textsubscript{2}O production in soil, nitrification and denitrification. This methodology is based on tracing stable isotopes of oxygen (O) and nitrogen (N) (\textsuperscript{18}O and \textsuperscript{15}N respectively) from enriched compounds (\textsuperscript{18}O water and \textsuperscript{15}N ammonium and/or nitrate) into N\textsubscript{2}O. Methodology based on \textsuperscript{15}N tracing has been well-established, but does not enable the distinction between N\textsubscript{2}O from nitrifier denitrification and nitrification, as the N\textsubscript{2}O-N is in both pathways derived from ammonium. The O in N\textsubscript{2}O originates both from O\textsubscript{2} and water (H\textsubscript{2}O). Reaction stoichiometry shows that the relative contribution of O\textsubscript{2} and H\textsubscript{2}O to the total O in N\textsubscript{2}O differs between the pathways of N\textsubscript{2}O production. The suggested dual isotope approach was based on the general understanding that next to reaction stoichiometry only isotopic fractionation would affect the O isotopic signal of N\textsubscript{2}O. The use of isotopically enriched compound enables to discard the latter effect and to distinguish the relative contributions of the production pathways based on tracing the \textsuperscript{18}O from H\textsubscript{2}O into N\textsubscript{2}O.

However, shortly after the start of my research I realized that there might be an additional defining factor of the O isotopic signature of the O in N\textsubscript{2}O: oxygen exchange between H\textsubscript{2}O and intermediates of the N\textsubscript{2}O production pathways. Throughout this thesis, ‘oxygen (or O) exchange’ is used as short for the exchange
of O between nitrogen oxides and H₂O. As a result of such exchange, relatively more H₂O-O could end up in the N₂O than based on reaction stoichiometry alone. Ignoring O exchange would distort the interpretation of the pathways’ relative contributions to N₂O production based on the O isotope signatures.

Against the background of the original main objective, but acknowledging the potential methodological constraints of the dual isotope approach caused by O exchange, the (revised) objectives of my PhD research have been:

(i) to study, identify and quantify the process of O exchange between H₂O and intermediates of the N₂O production pathways, and its effect on the O isotopic signature of N₂O from soil;
(ii) to develop and apply an advanced O and N isotope tracing approach that could distinguish nitrifier denitrification from ‘conventional’ nitrification and denitrification in soil-based studies; and
(iii) to evaluate the significance and idiosyncratic character of nitrifier denitrification as production pathway of N₂O in soil.

In chapter 2 I started my studies on O exchange by reviewing literature on the potential occurrence of O exchange. In many pure culture studies on both nitrifiers and denitrifiers, significant rates of O exchange have been reported. Although hardly considered in ecosystem studies, O exchange could therefore very likely be present in soil and aquatic environments. I concluded that the use of the O isotopic signature in source determination of N₂O, and potentially other nitrogen oxides as well, should therefore be adopted with extreme caution.

I subsequently studied the process of O exchange experimentally, on a variety of soils from across Europe. In chapter 3 I showed that O exchange can indeed strongly determine the O isotopic composition of N₂O. First, I identified O exchange by studying the incorporation of ¹⁸O from H₂O into N₂O. In all soils, the amount of O in N₂O derived from H₂O exceeded the amount that could be explained by reaction stoichiometry alone. Second, I quantified O exchange during denitrification of NO₃⁻ after examining the recovery of ¹⁸O from applied ¹⁸O enriched NO₃⁻ into the produced N₂O. In the absence of O exchange, the ratio of the ¹⁸O and ¹⁵N enrichment of NO₃⁻ should be retained in the N₂O. However, the ¹⁸O:¹⁵N enrichment ratio retention, the ‘ERR’, revealed that the enrichment of
the $^{18}$O has strongly declined relative to the $^{15}$N. With the loss in ERR I quantified that during the reduction of NO$_3^-$ to N$_2$O, up to 97% of the NO$_3^-$-O had exchanged with (non-enriched) O from H$_2$O.

The ERR approach quantified the O exchange for the pathway of denitrification. In chapter 4, I further examined the O isotopic signature of the N$_2$O from my labeling experiments to assess the potential presence of O exchange during the other N$_2$O production pathways. Assuming the presence or absence of O exchange under a series of scenarios, I evaluated the observed N$_2$O-O isotopic signature. This assessment revealed that the O exchange during the reduction of NO$_3^-$ alone could not fully explain the observed $^{18}$O enrichment of the N$_2$O: during other pathways of N$_2$O production, additional O exchange with H$_2$O must have occurred. Nitrifiers could thus mediate O exchange as well, during nitrifier denitrification, nitrite oxidation to nitrate, or both.

In chapter 5 I developed and applied an advanced dual isotope approach, with the aim to discriminate nitrifier denitrification from ‘conventional’ nitrification and denitrification as pathways of N$_2$O production in soil. This approach integrates the quantified O exchange during denitrification and anticipates on the additional presence of O exchange with $^{18}$O and $^{15}$N isotope tracing. The remaining uncertainty about the presence and extent of O exchange is controlled by adopting various assumptions. As a result, the contribution of the different nitrifier pathways (including nitrifier denitrification) could not be narrowed down to one number. However, it did enable to identify a minimum to maximum range of the contribution of nitrifier denitrification to the total N$_2$O production. With a minimum of zero, the presence nitrifier denitrification would not be conclusively proven. Yet, a minimum contribution of nitrifier denitrification (larger than zero) was quantified for multiple soils, and thus identified that nitrifier denitrification can indeed be a production pathway of N$_2$O in soils. In these experiments, the soils were studied under relative high moisture conditions (80% WHC). This likely explains why in those experiments total N$_2$O production was dominated by NO$_3^-$ driven denitrification. Consequently, the identified minimum contribution of nitrifier denitrification remained low for most soils (over 60% of total N$_2$O for one soil, but less than 10% for the others). However,
actual contributions may likely have been larger, as this minimum was defined under rather extreme assumptions. Moreover, assessment of the total nitrifier-derived N₂O revealed that the contribution of nitrifier denitrification strongly dominated over that of ‘conventional’ nitrification.

Environmental controls of N₂O production may likely affect the individual pathways differently. In chapter 5 I evaluated soil pH and C content as possible predictors of the relative pathway contributions to N₂O production. Relative contributions of NH₄⁺ and NO₃⁻ derived N₂O showed to be related with both soil pH and soil C content. However, with the small contribution of NH₄⁺ (i.e. total nitrifier contribution) to total N₂O and the ability to only partially quantify the different nitrifier pathways, the contribution of nitrifier denitrification was not distinct enough to evaluate its individual relation with the diversity in pH and soil carbon. However, both in chapter 5 and in my discussion I speculate how nitrifier denitrification may likely respond idiosyncratically to these soil parameters.

In chapter 6 I similarly studied the N₂O production pathways, this time on a single soil at three different moisture levels. Under slightly more moderate moisture conditions in these experiments (50% and 70% WHC), nitrifier denitrification was responsible for more of the total N₂O than ‘conventional’ denitrification of NO₃⁻. Nitrifier denitrification was shown to constitute a significant contribution to soil-derived N₂O. Moreover, with this experiment I demonstrated that nitrifier denitrification indeed responds idiosyncratically to soil moisture content. Compared to ‘conventional’ denitrification, nitrifier denitrification was less repressed by aerobic conditions. Under relatively moderate moisture conditions, N₂O production by nitrifier denitrification was likely equally significant as N₂O from ‘conventional’ nitrification.

Next to N₂O, also for nitrate (NO₃⁻) the O isotopic signature is commonly used to evaluate its sources and cycling in ecosystems. In chapter 7 I therefore carried out an exploratory study on the potential effect of O exchange on NO₃⁻. In this experiment I observed a decrease in the ¹⁸O enrichment of the NO₃⁻, while the ¹⁵N enrichment did not significantly change over the incubation period. This
demonstrates that O exchange might indeed affect the O isotopic signature of NO\textsubscript{3}\textsuperscript{-} in soil.

In conclusion, my studies established that O exchange between H\textsubscript{2}O and intermediates of N\textsubscript{2}O production processes is a defining factor of the O isotopic signature of N\textsubscript{2}O and probably NO\textsubscript{3}\textsuperscript{-} as well. This evidently constitutes implications for source determination studies of N\textsubscript{2}O and NO\textsubscript{3}\textsuperscript{-} that are based on the interpretation of the O isotopic signature. Taking the effect of O exchange into account, I developed a novel dual isotope tracing approach to study pathways of N\textsubscript{2}O production. Subsequently, my studies identified the presence, significance, and idiosyncratic character of nitrifier denitrification as production pathway of N\textsubscript{2}O in soil. The acknowledgement of nitrifier denitrification as distinct N\textsubscript{2}O production pathway in soil is an important step forward in our understanding of N\textsubscript{2}O production to ultimately enable the development of accurate inventories and effective mitigation strategies for N\textsubscript{2}O emissions.
Lachgas (N₂O) is een broeikasgas dat bijdraagt aan de opwarming van de aarde en de afbraak van ozon in de stratosfeer. Om emissies van N₂O terug te dringen zullen we moeten begrijpen hoe en waar het ontstaat. Er is echter veel onduidelijkheid over de verschillende manieren waarop N₂O wordt gevormd, en hoe deze processen worden beïnvloed door de omgeving. Wereldwijd vormen bodems de grootste bron van lachgas naar de atmosfeer. Met mijn onderzoek probeer ik daarom een beter inzicht te krijgen in de productie van N₂O in bodems. Mijn hoofddoel was om de bijdrage van ‘nitrifier denitrification’ als afzonderlijk proces te bestuderen. In dit proces wordt nitriet (NO₂⁻) omgezet in N₂O door ammonia oxiderende bacteriën (AOB), die normaliter juist NO₂⁻ vormen vanuit ammonia (NH₃). Het reducerende proces tot N₂O wordt normaal gesproken voornamelijk toegeschreven aan andere organismen, de denitrificeerders. ‘Nitrifier denitrification’ is in studies met reinculturen van AOB echter al enige tijd erkend. Maar doordat onderzoekstechnieken ontoereikend bleken is het tot op heden onduidelijk gebleven of dit proces ook in bodems plaatsvindt.

Recent is er een nieuwe methode ontwikkeld om nitrifier denitrification te onderscheiden van nitrificatie en denitrificatie, traditioneel de twee belangrijkst geachte N₂O vormende processen in de bodem. Deze methode bestudeert de stabiele isotopen van zuurstof (O) en stikstof (N) (respectievelijk ¹⁸O en ¹⁵N) in N₂O. Het ¹⁵N ‘signaal’ wordt al regelmatig gebruikt, maar kan niet het onderscheid tussen N₂O uit ‘nitrifier denitrification’ en nitrificatie maken, omdat voor beide processen de N afkomstig is van ammonia. De herkomst van de O in N₂O is wel verschillend voor deze processen en wordt volgens de reactievergelijkingen in verschillende verhoudingen geleverd door zuurstofgas (O₂) en water (H₂O). Verschil in het ¹⁸O signaal van O₂ en H₂O resulteert daarmee in verschil in het ¹⁸O signaal van N₂O. Volgens deze nieuwe methode zou daarmee de bijdrage van de verschillende processen aan de totale N₂O productie onderscheiden kunnen worden.

Echter, kort na de start van mijn onderzoek kwam ik tot de ontdekking dat nog een ander proces invloed kan hebben op het ¹⁸O signaal van N₂O: uitwisseling van O tussen H₂O en tussenproducten van de reacties die N₂O vormen, kortweg ‘zuurstof (of O) uitwisseling’. Door zuurstof uitwisseling kan er meer O van H₂O in N₂O terecht komen dan men zou verwachten op basis van de
reactievergelijking. Als dit effect wordt genegeerd zal de bijdrage van de verschillende processen aan de N\textsubscript{2}O vorming verkeerd worden geïnterpreteerd. Met aandacht voor de beperking van O uitwisseling voor de voorgestelde nieuwe methode werden de belangrijkste doelen van mijn onderzoek:

(i) het bestuderen van O uitwisseling tijdens de vorming van N\textsubscript{2}O in de bodem, en het aantonen en kwantificeren van het effect ervan op het O isotopen signaal van N\textsubscript{2}O;

(ii) het ontwikkelen van een aangepaste methode om met behulp van O en N isotopen onderscheid te maken tussen de N\textsubscript{2}O vormende processen in de bodem;

(iii) het in kaart brengen van de bijdrage en unieke karakter van ‘nitrifier denitrification’ als N\textsubscript{2}O vormend proces in de bodem.

Mijn onderzoek begint in hoofdstuk 2 met een literatuurstudie naar zuurstof uitwisseling. Verscheidene studies tonen aan dat O uitwisseling kan plaatsvinden met reincultures van zowel nitrificeerders als denitrificeerders. Hoewel er in studies in bodem en aquatische systemen nauwelijks rekening mee wordt gehouden, zou O uitwisseling dus wel degelijk kunnen voorkomen in natuurlijke systemen. Ik concludeerde da t men bij het gebruik van het \textsuperscript{18}O signaal om de bronnen van N\textsubscript{2}O te onderscheiden zeer alert moet zijn op het mogelijke effect van O uitwisseling.

Vervolgens bestudeerde ik O uitwisseling daadwerkelijk in experimenten met verschillende bodems. In hoofdstuk 3 laat ik zien dat O uitwisseling inderdaad een groot effect heeft op het \textsuperscript{18}O signaal van N\textsubscript{2}O. Ten eerste bewees de hoge verrijking van \textsuperscript{18}O in N\textsubscript{2}O na toevoeging van \textsuperscript{18}O-verrijkt H\textsubscript{2}O dat uitwisseling plaats moest hebben gevonden. Ten tweede kon ik de uitwisseling tijdens de omzetting van nitraat (NO\textsubscript{3}\textsuperscript{−}) naar N\textsubscript{2}O kwantificeren door \textsuperscript{18}O en \textsuperscript{15}N verrijkt NO\textsubscript{3}\textsuperscript{−} te gebruiken. Het verlies van \textsuperscript{18}O in verhouding tot \textsuperscript{15}N na de omzetting tot N\textsubscript{2}O liet zien dat in sommige gronden bijna alle \textsuperscript{18}O in nitraat was verwisseld voor (niet-verrijkte) O uit H\textsubscript{2}O.

Deze benadering stelde mijn in staat om O uitwisseling tijdens de omzetting van nitraat (NO\textsubscript{3}\textsuperscript{−}) naar N\textsubscript{2}O, ofwel denitrificatie, te kwantificeren. In hoofdstuk 4 beschrijf ik hoe het O signaal van de N\textsubscript{2}O verdere informatie geeft over O uitwisseling gedurende andere N\textsubscript{2}O vormende processen. Ik bereken voor
verschillende scenario’s waarin ik in meer of mindere mate rekening hou met O uitwisseling wat het verwachte $^{18}$O signaal van N$_2$O zou zijn, en vergelijk dit met het gemeten signaal. Dit liet zien dat O uitwisseling niet alleen tijdens denitrificatie moet hebben plaatsgevonden, maar ook gedurende nitrificatie en/of nitrifier denitrification.

In hoofdstuk 5 gebruik ik de opgedane kennis over O uitwisseling om een aangepaste methode te ontwikkelen die met gebruik van $^{18}$O en $^{15}$N alsnog N$_2$O productie uit nitrifier denitrification kan onderscheiden van nitrificatie en denitrification. Omdat ik daarnaast nog een aantal aannames moet doen kan de bijdrage van nitrifier denitrification niet exact worden gekwantificeerd, maar wel met een marge (een minimum en maximum). Met deze vernieuwde aanpak bestudeerde ik 12 verschillende Europese gronden. De minimum bijdrage van nitrifier denitrification aan N$_2$O productie was in meerdere van deze gronden groter dan nul. Met andere woorden: hier toon ik voor het eerst aan dat nitrifier denitrification inderdaad plaats kan vinden in de bodem. Over het algemeen leek de relatieve bijdrage van nitrifier denitrification klein ten opzichte van klassieke denitrificatie, maar het was duidelijk hoger dan N$_2$O productie uit nitrificatie.

Factoren die bepalend zijn voor de productie van N$_2$O beïnvloeden de afzonderlijke processen wellicht in verschillende mate en op verschillende manieren. In hoofdstuk 5 onderzoek ik daarom ook of de pH en het koolstof (C) gehalte van de bodem de verschillen in de bijdrage van de afzonderlijke processen kan verklaren. De relatieve bijdrage van ammonium (NH$_4^+$) en nitraat (NO$_3^-$) bleken gerelateerd aan de pH en het C gehalte van de bodem. Echter, de totale bijdrage van NH$_4^+$ (nitrificatie en nitrifier denitrificatie) bleef zoals gezegd klein, waardoor de specifieke bijdrage van nitrifier denitrification niet groot genoeg was om een relatie met pH en/of C verder te beoordelen.

In hoofdstuk 6 bestudeer ik op vergelijkbare wijze de lachgasproductie, maar dit keer gebruikte ik slechts één grond om het effect van verschil in vochtgehalte te onderzoeken. Het hoge vochtgehalte in voorgaande experimenten was wellicht de oorzaak van de relatief kleine bijdrage van NH$_4^+$, en dus van nitrificatie en nitrifier denitrification. In de bodems in dit experiment die iets minder vochtig waren was de bijdrage van nitrifier denitrification aan de N$_2$O productie beduidend groter, en belangrijker dan klassieke denitrificatie. Dit toont ook aan dat nitrifier
denitrification idiosyncratisch (dus op unieke wijze) beïnvloed wordt door omgevingsfactoren zoals vochtgehalte: onder (relatief) drogere omstandigheden nam N₂O productie door klassieke denitrificatie van NO₃⁻ veel sterker af dan productie via nitrifier denitrification. Onder dergelijke omstandigheden was de bijdrage aan de totale N₂O productie van nitrificatie en nitrifier denitrification (beide afkomstig van NH₄⁺) van vergelijkbare grootte, terwijl nitrificatie verwaarloosbaar was ten opzichte van nitrifier denitrification onder het hoge vochtgehalte.

Het O isotopen signaal wordt niet alleen gebruikt om de oorsprong van N₂O te bepalen; dit gebeurt ook voor nitraat (NO₃⁻) in bijvoorbeeld oppervlakte- en grondwater. In hoofdstuk 7 presenteer ik daarom een verkennende studie naar een mogelijk effect van zuurstof uitwisseling op het zuurstof isotopen signaal van NO₃⁻. In dit experiment constateerde ik dat het ¹⁸O signaal van NO₃⁻ in de bodem significant was afgenomen na 24 uur, terwijl het ¹⁵N signaal onveranderd was. Dit impliceert dat zuurstof uitwisseling inderdaad ook bepalend zou kunnen zijn voor het isotopen signaal van NO₃⁻.

Samengevat laat ik met mijn onderzoek zien dat uitwisseling van zuurstof tussen water en tussenproducten van lachgasproductie een belangrijke factor is in het bepalen van het uiteindelijke zuurstof isotopen signaal van lachgas (N₂O), en wellicht ook van nitraat (NO₃⁻). Dit heeft gevolgen voor onderzoek naar de herkomst van N₂O en NO₃⁻, omdat het zuurstof isotopen signaal daarin vaak gebruik wordt als indicator. Ik introduceerde een nieuwe methode om lachgasproductie te bestuderen waarin het effect van zuurstofuitwisseling expliciet wordt meegenomen. Daarmee heb ik ‘nitrifier denitrification’ als N₂O vormend proces kunnen onderscheiden van nitrificatie en denitrificatie, en voor het eerst kunnen aantonen dat ook ‘nitrifier denitrification’ een belangrijke bijdrage kan leveren aan de N₂O emissie uit de bodem. Ook liet ik zien dat ‘nitrifier denitrification’ anders wordt beïnvloed door omgevingsfactoren dan andere N₂O vormende processen. De bevestiging dat nitrifier denitrification een afzonderlijk en belangrijk proces van lachgasproductie in de bodem is draagt bij aan ons begrip van de herkomst van lachgas. Inzicht in de vorming van lachgas is essentieel om uiteindelijk de emissies van dit broeikasgas effectief terug te kunnen dringen.
Dankwoord
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Curriculum vitae and publications
Curriculum vitae

Dorien Kool was born in Oosterhout, the Netherlands, on September 6th 1982. She completed her secondary (VWO) in 2000, and in the same year she moved to Wageningen to study ‘Soil, Water and Atmosphere’. She specialized in soil science for her BSc. For her first MSc thesis project she studied the oxidation and compaction of tropical peat land in Kalimantan, Indonesia, in cooperation with the BOS Foundation. She worked at Alterra (Wageningen) for her second MSc thesis, on emissions of nitrous oxide (laughing gas) from cattle urine deposition. To finalize her studies she did her internship at the University of California in Davis, where she did research on soil carbon saturation under elevated levels of atmospheric CO₂. In March 2006 she received her MSc degree (cum laude) in Soil Science and in Earth System Science. Caught by the ironically serious subject of her second MSc project Dorien started her PhD research on nitrous oxide. During her PhD she received the Best Publication Award 2009 of graduate school PE&RC for her publication in Rapid Communications in Mass Spectrometry, and the Schulzman Award for her presentation at the AGU Fall Meeting 2009.

List of publications


PE&RC Education Certificate
PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- Microbial sources of N₂O: the contribution of nitrifier denitrification (2006)

Writing of project proposal (4.5 ECTS)
- Microbial sources of N₂O: the contribution of nitrifier denitrification (2006)

Post-graduate courses (6 ECTS)
- NitroEurope summer school ‘Microbial nitrogen turnover and production of greenhouse gases’, Vienna, Austria (2006)
- Advanced statistics, Wageningen, the Netherlands (2007)
- Postgraduate course ‘Biodiversity and ecosystems services’, Wageningen, the Netherlands (2008)

Laboratory training and working visits (4.5 ECTS)
- Visit to Dr. N. Wrage, University of Gottingen, Germany (2008)
- Visit to Dr. D. Harris, Stable Isotope Facility, University of California-Davis, USA (2008)
- Visit to Dr. T. Clough, Lincoln University, New Zealand (2010)

Invited review of (unpublished) journal Manuscript (7 ECTS)
- Reviewer of seven articles submitted to international scientific journals Soil Biology & Biochemistry, Rapid Communications in Mass Spectrometry, Geoderma, Global Change Biology (2007-2010)

Deficiency, refresh, brush-up courses (1.5 ECTS)
- Basic statistics (2006)
Competence strengthening, skills courses (6.4 ECTS)
- PhD competence assessment (2006)
- MSc thesis supervision (2008)
- Communicating your science to the public (2008)
- NWO talent day (2009)
- Teaching methodology and skills for PhD students (2009)
- PhD course ‘Mobilizing your scientific network’ (2009)
- PhD course ‘Career perspectives’ (2009)
- NWO talent class ‘Subsidies aanvragen’ (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)
- PE&RC days 2007-2009

Discussion groups, local seminars, other scientific meetings (6.4 ECTS)
- CPN discussion group (2006-2007)
- PE&RC discussion group Climate change & soil-water-vegetation interaction (2007-2010)

International symposia, workshops and conferences (25.3 ECTS)
- Annual meetings of the NitroEurope IP; oral presentations (2006 - 2010).
- DIARP workshop, Wageningen, the Netherlands; oral presentation (2007)
- 15th Nitrogen workshop, Lleida, Spain; oral presentation (2007)
- 13th Meeting of COST-action on denitrification, Llubjana, Slovenia; poster (2007)
- ASA-CSSA-SSSA annual meeting, New Orleans (LA), USA; oral presentation (2007)
- ISOEcol 6, Honolulu (HI), USA; poster (2008)
- AGU Fall meeting 2008, San Francisco (CA), USA; oral presentation (2008)
- Soil Organic Matters meeting, Rothamsted, United Kingdom; oral presentation (2009)
- AGU Fall meeting 2009, San Francisco (CA), USA; oral presentation (2009)

Lecturing, supervision of practical’s, tutorials (2.4 ECTS)
- BSc course ‘Biological interactions in soils’, practical supervisor (2008)
- BSc thesis supervision, daily supervisor (2009)